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BACTERIOLOGICAL STERILITY OF TISSUES DERIVED FROM SECONDARY CROWN-GALL TUMORS

ARMIN C. BRAUN AND PHILIP R. WHITE

(Accepted for publication May 27, 1942)

INTRODUCTION

Phytomonas tumefaciens (Smith and Town.) Bergey *et al.*, upon inoculation into sunflower, Paris daisy, and probably other hosts as well, induces not only the production of primary tumors at the site of inoculation but also secondary tumors at points distant from the primary foci (2, 6, 9). These secondary tumors bear a superficial resemblance to the metastatic growths found in certain malignant animal neoplasia. The distribution of secondary growths, together with the morphological and cytological similarities of both primary and secondary crown-gall tumors to certain tumorous growths of animals and of man, has led to their frequent inclusion in discussions of neoplasia in general.

Erwin F. Smith, who studied the crown-gall problem intensively for more than two decades, believed that *Phytomonas tumefaciens* was intimately bound up with the host cells, multiplied as the host cells multiplied, and was directly responsible for the continued development of the tumor. Most other workers in the field have concurred in this view, although not always agreeing with Smith as to the exact position of the bacteria in the host tissue.

Although *Phytomonas tumefaciens* is known to initiate tumor formation in plants, this organism has not always been isolated from old primary tumors. It can seldom be obtained from secondary tumors in the sunflower even after special effort. This has led certain workers to question the necessity of the continued presence of bacteria for the continued proliferation of the host tissue and to postulate that the host cells involved may sometimes acquire a capacity for autonomous growth.

Studies on the question of autonomy of crown-gall cells have been complicated in the past by the fact that sterile tumor tissue was not readily available. Primary tumors usually contain, in addition to the causal agent of the disease, saprophytic organisms of many kinds. Recently, however, the writers of this paper have submitted evidence (8, 9) that bacteria-free tumor tissue can be secured from the nodular secondary tumors of sunflower plants, and that this tissue is capable of autonomous neoplastic growth both *in vivo* and *in vitro*.

Clones of tissue cultures isolated from 10 metastatic tumors were subjected to careful study. Although derived from different tumors, all showed similar morphological and cytological characteristics which differentiated them sharply from cambial or procambial tissue cultures isolated from healthy sunflower plants. The normal plant tissues grew in culture very

slowly, were usually green in color, and often developed roots. They showed considerable internal structural organization, evidencing a fairly pronounced response to the morphogenetic restraints characteristic of the same tissues when growing *in situ*. The tumor-tissue cultures, on the other hand, showed a high degree of independence. They grew very rapidly, were glistening white, and never developed roots. Internally they were composed mostly of parenchymatous tissue with scattered scalariform elements that never became organized into any coherent system. While cultures of healthy sunflower tissue increased in volume about 250 times in slightly more than a year, the tumor-tissue cultures showed a theoretical increase of a hundred million million times during the same period. Moreover, these tumor tissues retained undiminished their tumor-inducing capacity for more than a year. The tissue has been successfully transplanted from sunflower to the closely related Jerusalem artichoke and back again to sunflower plants, producing characteristic tumors in both hosts. The gross structure and histological picture presented by these transplant tumors were similar to those of the galls produced by *Phytomonas tumefaciens*. No tumors were ever produced when cultures of healthy tissues were similarly treated.

The cells of secondary crown-gall tumors behave, then, like malignant animal cells in several respects. They maintain an uncontrolled growth in the initial host. They can be transplanted successfully to other plants of the same or of closely related species without change in behavior. They retain these characteristics of uncontrolled autonomous growth, probably indefinitely, when grown both *in vivo* and *in vitro*.

It is the purpose of this paper to outline in detail the evidence upon which is based the conclusion that these autonomous and potentially malignant tissues are, in fact, free of *Phytomonas tumefaciens*.

METHODS

Most of the present study is based on tissue cultures isolated from the nodular secondary tumors developed above the site of the primary tumor on sunflower plants (Fig. 1). Plants of sunflower (*Helianthus annuus* L. var. Giant Russian) were grown in compost soil in 4-inch pots and were kept on the greenhouse bench throughout the course of the experiments. When they had reached a height of 9 to 12 inches, they were inoculated with the highly virulent A_6 strain of *Phytomonas tumefaciens*. Inoculations were always made by needle puncture into an internode at a distance of not less than 3 inches from the apical bud. Primary tumors developed in all inoculated plants, followed in about 60 per cent of the cases by metastatic secondary tumors. It was felt that a thoroughgoing attempt to obtain bacteria from secondary-tumor tissues or evidence of the presence of bacteria in such tissues should be made.

When the nodular secondary tumors had grown to the size of a small pea, usually 5 to 6 weeks after inoculation, they were sacrificed. Tissue from such growths was isolated and grown *in vitro* according to methods described elsewhere.

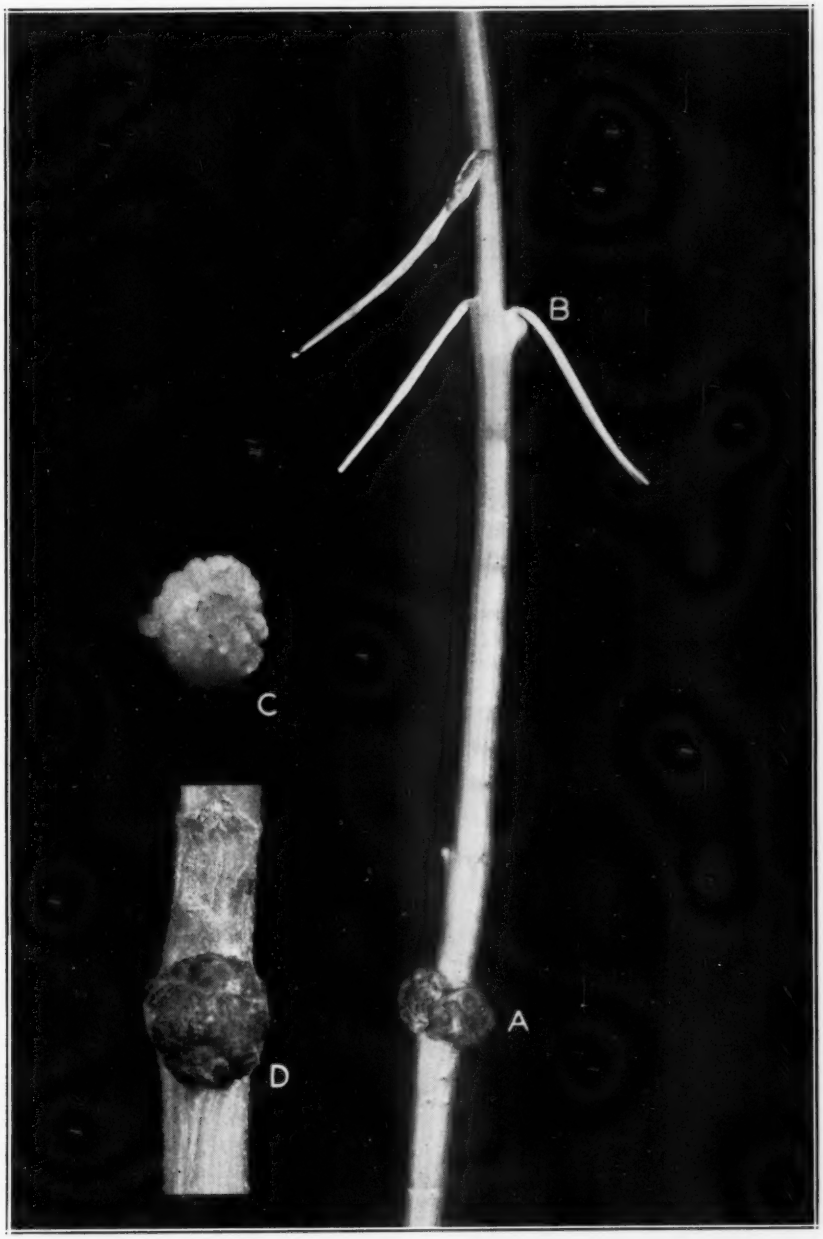

FIG. 1. A. Primary tumor formed at the point of inoculation in a sunflower plant. B. Nodular secondary tumor located 2 nodes above the primary tumor. The tissue composing this type of nodular overgrowth is generally free of bacteria. C. Bacteria-free tumor-tissue culture originally derived from a fragment of a secondary tumor similar to that shown at B. D. Tumor produced on a sunflower stem by the implantation and growth of a small fragment of a bacteria-free tumor-tissue culture. Note the similarity in structure between the bacteria-free tumor at D and the bacteria-containing tumor at A. (Photograph by J. A. Carlile.)

Bacteriological tests were applied to: (1) tissue freshly isolated from various regions of infected plants; (2) tissue cultures derived from one secondary tumor after 5, 10, 15, 20, and 25 passages *in vitro* and at various miscellaneous intervals between, tissue cultures derived from one additional tumor after 16 passages *in vitro,* and tissue cultures from 8 other secondary tumors after 9 passages *in vitro;* (3) tissue isolated from second-generation tumors produced by implanting tissue cultures in new hosts. These totaled about 1250 tests. Serological tests were applied to tissue cultures of the first group mentioned above after they had been maintained for 10 or more passages *in vitro.* These employed 125 cultures. About 1000 tissue cultures, mostly of this first group, were maintained for periods of 2 weeks or more growing on a nutrient suitable for the isolation of crown-gall bacteria. Grafting experiments in which about 700 tissue cultures were used constitute a 4th type of test the results of which bear on the question of the bacteriological sterility of secondary tumors and materials derived therefrom. Additional special tests also were made on a few tissue cultures and will be outlined later. In all, the results of a total somewhat more than 2000 tests of various kinds are included in the present paper.

RESULTS

Bacteriological Tests

Recovery of *Phytomonas tumefaciens* from certain types of tumor tissue is considered difficult, and the need for applying suitable methods when attempting to isolate this organism has frequently been emphasized. Crown-gall bacteria are considered present in pockets scattered throughout the tumor tissue. Success in isolation would therefore appear to depend in large measure on the method used to release the bacteria from these pockets. A thorough trituration of the tissue is obviously important. This was accomplished either by tearing with sterile needles, by grinding the material in a sterile mortar, or by crushing it in a sterile test tube with the aid of a glass rod. A few milliliters of nutrient-dextrose broth, potato-dextrose broth, or yeast-water-mannitol broth were added before crushing the tissues. The thoroughly triturated tissue was permitted to incubate at room temperature for periods ranging from 1 to 8 hours or longer. The tissue suspensions were then pipetted into sterile Petri plates and the desired agar media added. The plates were rotated in the usual manner to insure uniform distribution of the tissue. All plates were incubated at 25° C. for at least 3 weeks before being discarded.

Bacterial Flora of Tissue Freshly Isolated from Various Regions of Infected Plants. Fragments of tissue were excised aseptically from various regions of sunflower plants carrying well established infections of *Phytomonas tumefaciens.* These were triturated in media suitable for the isolation of this organism. Plates were incubated either until crown-gall organisms appeared or until they had remained sterile for at least 3 weeks. The results are shown in table 1. Primary tumors were 100 per cent positive. The

incidence of crown-gall bacteria decreased rapidly from the primary tumors upward so that, while secondary tumors at the first node above the primary were positive in 83 per cent of the 35 cases tested, tumors at higher sites were positive in only 4 per cent of the 181 cases tested. Apparently healthy tissues showed almost as high an incidence of infection, indicating that this 4 per cent might be looked upon as due to chance contaminants having no clear causal relation to the incidence of tumors. Secondary tumors at distances of more than one node from the primary were free of bacteria in by far the majority of cases.

This observation, although contrary to the usually accepted idea, is entirely consonant with Erwin F. Smith's observations. In his 1912 paper (7) he records, for plant No. 1 (Paris daisy), 20 plates from 3 secondary tumors and 21 plates from 3 "tumor strands" all negative. From plant VII he records an unspecified number of plates from 4 secondary tumors and 7

TABLE 1.—*Incidence of crown-gall bacteria in different regions of infected sunflower plants*

	Primary tumors	Secondary tumors		Apparently healthy tissues at a distance of 6–12 inches above the primary tumor	Total
		Located at 1st node above primary tumor	Located at 2nd, 3rd, and 4th nodes above primary tumor		
Number tested	100	35	181	100	416
P. tumefaciens isolated from	100	29	7	2[a]	.....
Per cent infected with *P. tumefaciens*	100	83	4	2	.....

[a] Each plate contained a single colony of *P. tumefaciens*.

tubes from 2 tumor strands, likewise all negative. From plant XIV he records an unspecified number of plates from a single ruptured tumor of which "a few" were positive. Specific tests from other plants were not recorded. Out of 13 secondary tumors and tumor strands tested, 12 thus appeared to be bacteria-free. While Smith attributed this to faulty technique and to the refractory nature of crown-gall bacteria, he did so apparently on the unproved assumption that tumefaction can occur only in the presence of bacteria. The actual observations are equally consonant with the alternative interpretation that the bacteria were not and had not been present in these regions.

Bacterial Flora of Tissue Cultures from Secondary Tumors. The methods used in isolating tissue for *in vitro* cultivation were similar to those employed for the isolation of crown-gall organisms except that the tissues were not triturated. Fragments of tissue were excised under aseptic precautions. No disinfectant of any kind was employed. The epidermis was first stripped off carefully. Tissue was then removed with a sharp scalpel

or a surgeon's ear curette. The fragments were placed on a semi-solid nutrient suitable for the growth of both excised plant tissues and crown-gall organisms. This consisted of White's glycine-thiamin-sucrose nutrient-salt solution to which 0.6 per cent of thoroughly leached agar was added. Fifty ml. of this nutrient was placed in 125-ml. Erlenmeyer flasks. Cultures that, in the first passage, developed contaminations either of *Phytomonas tumefaciens* or of air-borne or water-borne saprophytes (see above) were discarded, and only those that appeared sterile were used for further study. Cultures were divided at the end of each 2-week period until clones of the desired number of cultures were built up. Clones of tumor tissue were thus established from 10 secondary tumors. Eight clones have been carried for more than 9 passages, one clone for more than 16 passages, and another clone is now (May, 1942) in the 30th passage. Cultures have remained for a minimum of 2 weeks in each passage in intimate contact, both at intact and at freshly cut surfaces, with a nutrient capable of supporting growth of crown-gall organisms. More than 1000 cultures have been so maintained. In all these tests, *P. tumefaciens has, after the first passage, never appeared on a single culture.*

The nutrient used, however, has a pH reading of about 5.5. This is below the optimum for the initiation of growth by very small numbers of the crown-gall organism. It was important, therefore, to extend these tests by the use of special methods better adapted to the detection of small numbers of organisms or of organisms in a semi-dormant state. A method for this work was developed as follows.

Pure cultures of *Phytomonas tumefaciens* were streaked on nutrient agar plates and incubated at 25° C. for 21 days, so as to bring them into a negative and, hence, semi-dormant growth phase approximating the condition postulated for the supposed bacteria in the tumor tissues. Organisms were then transferred from these aged cultures into sterile tap water and shaken vigorously to break up the bacterial clumps. Dilutions were made and varying numbers of bacteria were introduced into the various broth media to be tested. To simulate the effects that the presence of sunflower tissue might have on the initiation of bacterial growth, a small, finely ground, tumor-tissue culture was added to each tube of broth under aseptic conditions. For every tube inoculated with bacteria and tumor tissue, 2 tubes were inoculated with tumor tissue alone as controls.

In preliminary tests, tubes inoculated with tumor tissue alone never produced growth that could be identified as *Phytomonas tumefaciens.* Tubes of nutrient-dextrose broth plus 0.1 per cent agar containing tumor tissue to which bacteria in numbers of 10 or more were added regularly produced bacterial growth. This medium is therefore *suitable* for the initiation of growth from as few as 10 viable bacterial cells in the presence of sunflower tissue. It, therefore, was used in most subsequent experimental work. Potato-dextrose broth and yeast-water mannitol broth also were found to favor growth from small numbers of bacteria but were employed to a lesser extent.

TABLE 2.—*Occurrence of molds and bacteria in tissue cultures from secondary crown-gall tumors*

Date	Tissue culture clone designation	Passage	No. of cultures	Medium used	Molds	Bacteria	Bacteria producing tumors on tomato plants	Percentage of cultures carrying crown gall
5/30/41	H 7	8	4	Nut. dext. agar	0	0	0	0
"	H 13	5	4	Nut. dext. agar	0	0	0	0
6/6/41	H 7	9	4	Nut. dext. agar	0	0	0	0
7/18/41	Not recorded	–	5	Nut. dext. agar	0	0	0	0
"	" "	–	5	Pot. dext. agar	0	0	0	0
"	" "	–	5	Patel's medium	0	0	0	0
"	" "	–	5	Nut. dext. broth	0	0	0	0
"	H 7	10	24	Pot. dext. agar	10[a]	[a]	0	0
"	"	10	76	Nut. dext. agar	8[a]	[a]	0	0
9/26/41	"	15	33	Nut. dext. agar	1	6	0	0
"	"	15	33	Pot. dext. agar	0	2	0	0
"	"	15	34	Patel's medium	1	1	0	0
12/2/41	"	20	33	Nut. dext. agar	1	2	0	0
"	"	20	33	Pot. dext. agar	3	0	0	0
"	"	20	34	Nut. dext. broth	1	2	0	0
12/5/41	H 13	16	14	Nut. dext. agar	0	0	0	0
"	"	9	11	Pot. dext. agar	0	0	0	0
"	1	9	5	Nut. dext. agar	1	0	0	0
"	2 A	9	3	Nut. dext. agar	0	2	0	0
"	"	9	2	Pot. dext. agar	0	1	0	0
"	4	9	3	Nut. dext. agar	0	0	0	0
"	"	9	2	Pot. dext. agar	0	0	0	0
"	8	9	5	Nut. dext. broth	0	0	0	0
"	10	9	3	Nut. dext. agar	0	0	0	0
"			2	Pot. dext. agar	0	0	0	0
"	20	9	5	Nut. dext. agar	0	0	0	0
"	22 A	9	5	Nut. dext. agar	4	1	0	0
"	22 B	9	5	Nut. dext. agar	0	3	0	0
"	H 7	13	314	Nut. dext. agar	3	32	0	0
2/27/42	"	25	96	Yeast-water-mannitol agar	6	0	0	0
Total	.....	.....	807	.....	39	52	0	0

[a] Bacteria and molds were not distinguished in the record of this group.

In any bacteriological study contaminations do, of course, occasionally occur. Bacterial colonies occasionally appeared on plates poured from broth inoculated with tumor tissue. When this happened, colonies from such plates were always reinoculated into young sunflower or tomato plants, even when their appearance showed clearly that *Phytomonas tumefaciens* was not their major constituent. When crown-gall organisms are inoculated into these hosts, tumors are always produced so that such a test furnishes a positive means of identifying the bacteria if they are *P. tumefaciens*.

Tests on tumor-tissue cultures, using these methods, gave the results shown in table 2. Out of 807 cultures tested 91 were contaminated, 39 with molds and 52 with bacteria. All bacteria, however, proved to be secondary invaders not identifiable as *Phytomonas tumefaciens*, since in no case did they produce tumors when inoculated into young tomato plants. Six per cent of the tumor-tissue cultures tested were contaminated with bacteria. While this appears to be a relatively high percentage of contamination, it should be recalled that during trituration the tissues often remained exposed to the laboratory air for several minutes. We believe that in some instances, at least, contamination resulted during this period of exposure. This belief seems justified by the fact that frequently only one bacterial or mold colony appeared on the plates. In at least 3 instances, however, the tissues apparently became contaminated during cultivation in the tissue-culture flasks. On December 5, 1941, one group of old cultures of the H 7 clone used in most of this work was suspected, because of the slimy appearance of some of its members, of harboring bacteria. These cultures represented a group originally set aside for some special experiments and left in the flasks without being transferred for 4 to 6 weeks instead of being transferred at 2-week intervals. The 314 cultures comprising this group were, therefore, all tested bacteriologically. Thirty-two were found contaminated. Of these, all contained the same large, slow-growing, spore-forming bacterium. It appears likely that this organism had been introduced into a single culture at an earlier date and that it had become spread throughout this group during the division and transfer of the contaminated tissue. Similar results were obtained when the 2 A and 22 B clones, each consisting of 5 cultures, were tested.

Nutrient-dextrose, potato-dextrose, and yeast-water-mannitol broths containing 0.1 per cent agar and finely ground tumor tissues have been shown by us to permit the initiation of growth by very small numbers of bacterial cells. These broths were, therefore, used in still further checks on the presence of *Phytomonas tumefaciens* in the tissues. Thirty tumor-tissue cultures were ground separately in sterile mortars, each with a few ml. of the desired broth. The tissue suspensions were then pipetted under aseptic conditions into test tubes containing either nutrient-dextrose, potato-dextrose, potato-dextrose plus 1 per cent yeast water, or yeast-water mannitol broths. Some of the tubes of each set were so slanted as to expose a large surface of the liquid to the air; others were kept upright and, consequently, had

only a small surface exposed. The results after a 6-week incubation period at 25° C. showed all but 3 of the 131 tubes sterile. One of the potato-dextrose-broth tubes was contaminated with mold, while 2 of the nutrient-dextrose-broth tubes were contaminated with a slow-growing rod-shape organism that formed long chains in the broth. These bacteria were inoculated to tomato plants, but no tumors resulted. The plants were discarded after 4 weeks.

Since the conventional methods of isolating *Phytomonas tumefaciens* failed to demonstrate the presence of this organism in the tumor-tissue cultures, still another method was tried. Ten tumor-tissue cultures were cut into sections about 100 μ in thickness. These tissue slices were aseptically transferred to solidified nutrient-dextrose agar and potato-dextrose agar in Petri plates. Bacteria-containing primary-tumor tissues from sunflower plants were similarly treated. The 50 slices of primary-tumor tissues yielded cultures of the crown-gall organism in every instance, while all of the more than 200 slices of the secondary-tumor tissue cultures remained sterile. These sterile tissue slices, in many instances, grew quite well on the potato-dextrose agar, and after a period of one month typical masses of tumor tissue were found.

Bacterial Flora of Second Generation Galls Produced on Sunflowers by Implantation of Tumor-tissue Cultures. One of the procedures used in studying the autonomy of tissue cultures has been the implantation of pieces of tissue under the bark of young healthy sunflower plants, either as small masses containing viable tumor cells or as triturated débris. Since the bringing of bacteria or bacteria-containing tissue in contact with a wounded susceptible surface of a suitable host plant regularly results in the production of a tumor, every graft of this sort that *fails* to produce a tumor constitutes evidence of the bacterial sterility of the tissue used. The converse is, however, *not* true, as we have shown elsewhere (10). Out of a total of about 800 such grafts carried out in the past year, about 650 have produced no tumors, showing that the tissues used were free of demonstrable tumefacient agents and, hence, free of viable crown-gall organisms.

Tumors are, as we have shown elsewhere, produced in a considerable percentage of cases upon implantation of tumor-tissue cultures. Tissue taken from such a second-generation tumor was ground in beef-extract peptone-dextrose broth, incubated for 2 hours, and then plated (5 plates) on beef-extract peptone-dextrose agar. One plate developed a spreader type of bacterial colony (not *Phytomonas tumefaciens*). The other 4 remained sterile. Eleven isolations of tumor tissue were made from a second old and partly necrotic second-generation tumor and placed on White's glycine-thiamin agar nutrient. Six developed bacterial contaminants, but none of these produced tumors on inoculation into tomato plants.

These results, although meager, are consistent with the rest in demonstrating that second-generation tumors as well as most secondary tumors and all tissue cultures are free of crown-gall bacteria by all bacteriological methods used.

Serological Tests

Since bacteriological tests by several methods failed to reveal crown-gall organisms in tumor-tissue cultures derived from secondary tumors, serological tests were applied as a further check on the presence of the crown-gall bacteria in these tissues. Rabbits weighing about 2000 g. were used. All were bled at the beginning of the experiment and their sera shown to be free of agglutinins for the strain of *Phytomonas tumefaciens* used in this work. Two rabbits were injected at spaced intervals with tumor tissues that had been maintained *in vitro* for at least 15 passages. As checks, 2 additional rabbits were injected with primary-tumor tissue that was about one month old and was known to contain the crown-gall organism. The antigens used for injection in both cases were prepared by thoroughly grinding 3 g. of tumor tissue together with sterile saline (0.85 per cent NaCl) in a mortar. The suspensions were then filtered through a 60-mesh wire sieve and injected intraperitoneally in 5-cc. doses, except for the first injection in which 2 cc. were used. Twelve injections were given at 4-day intervals and the rabbits were bled 8 days after the last injection. Trial bleedings revealed that the maximum titre against the crown-gall organism in the bacteria-containing tumor tissue was reached after the 9th injection, no increase in titre being observed during the last 3 injections. A saline suspension of a 36-hour nutrient dextrose agar culture was used as the test antigen. Agglutination tests showed that the sera of rabbits injected with the bacteria-containing primary tumor tissues had titres against the A_6 strain of *P. tumefaciens* of 1–180 and 1–320, respectively. The other two sera, prepared by injecting ground-up tumor-tissue cultures into rabbits, did not react against the crown-gall organism even at serum dilutions as low as 1 : 1. Complement-fixation tests in which formolized bacillary suspensions of the A_6 strain of *P. tumefaciens* were used as test antigens fully confirmed the results of the agglutination tests.

Special Tests

The Question of Filterable Forms. The possibility that some morphological variant of *Phytomonas tumefaciens* other than the normal rod-shape organism may have been present in the tissues must also be considered. Levine (5), working with 3-month-old cultures of this organism, observed amorphous masses of protoplasm similar to the symplasm described by other workers. He also found in these cultures minute spherical bodies, faintly-staining cocci, and slender filamentous elements. These observations, he believed, supported the idea of there being a life cycle in these bacteria. D'Herelle and Peyre (4) suggested that a filterable probacterial form, rather than the normal rod-shape organism, might actually be the true cause of the crown-gall disease. They reported that filtered extracts from sugar-beet galls were capable of inducing tumors on healthy beets. The normal rod-shape organism was, however, always isolated from the latter. Almon and Baldwin (1), working with the closely related organisms, *Rhizobium trifolii*

Dangeard and *R. leguminosarum* Frank, found filterable forms present in a large majority of cultures that had aged for periods of one month or longer. Agglutination tests indicated some group relationship between the aberrant types and the normal forms. The aberrant types that developed from the filterable forms were, however, noninfectious on their respective host plants. Other reports recently published indicate that filterable forms may be present in other bacterial species.

Perhaps the most widely used and the most satisfactory method thus far developed for the cultivation of filterable forms is the Hauduroy technique (3). A modification of this method was used. Three tissue cultures from secondary tumors carried through 10 passages *in vitro* were ground separately, each in 5 ml. of sterile nutrient broth. The tubes were permitted to incubate at room temperature for one month. No visible bacterial growth appeared in any of the tubes. Three additional tumor-tissue cultures were then ground in a similar manner and were incubated at room temperature for 48 hours. These likewise remained sterile. Portions consisting of 0.5 ml. of broth from each of the 3 tubes that had been incubated for one month were added separately to 3 flasks containing yeast-water-mannitol agar, potato-dextrose agar, and nutrient-dextrose agar. The second series of tubes, which contained tumor-tissue cultures ground and incubated for 48 hours, were treated in a similar manner. The flasks were incubated at 25° C. for one week, after which time fresh portions of 0.5 ml. of broth were added to each of the flasks and the washings transferred to fresh agar of the same composition as that used for the original flasks. Twelve successive passages on this nutrient were made in 5 of the 6 cases. One of the flasks containing the potato-dextrose agar became contaminated with mold during the 5th washing. At the end of the 12th passage, none of the 5 remaining flasks showed any visible evidence of bacterial growth. Microscopic examination of the clear washings failed to reveal the presence of any recognizable bacterial elements. The results presented make it unlikely, therefore, that any filterable or otherwise aberrant form of the crown-gall organism was present in, and in any way a significant factor in the development of, the secondary tumor tissues grown *in vitro*.

Plant Inoculation Studies

Plant-inoculation tests were made as an additional check on the presence of the normal or aberrant forms of *Phytomonas tumefaciens*, as well as on the presence of other agents that might be involved in inciting the continued abnormal proliferation of the cells of tumor-tissue cultures. Two methods of introducing the tissues into the host plants were used. The first of these consisted of grinding the tumor-tissue culture and placing the crushed material in wounds in the stems of sunflower plants. The wounds were tightly bound with sterilastic tape in a manner similar to that used when fragments of tumor-tissue cultures were grafted in the host (9). Out of 200 tests made in this way, all except one healed without significant deviation from normal

wound response. In this one instance a small slow-growing tumor developed. After it had reached the size of a pea, the tumor was crushed in the usual manner and tested for its possible bacterial content by plating in Patel's medium. *P. tumefaciens* was not isolated and the plates were discarded at the end of 4 weeks. That the tumor did not arise from a chance contamination with *P. tumefaciens* is indicated by the localized growth, which is in marked contrast to the diffuse tumefactions that arise when large wounds are thus contaminated. It is believed probable that a small fragment of the tumor tissue had not been sufficiently crushed to destroy all viable tumor cells and that the subsequent growth of these residual cells resulted in the formation of the small tumor.

The second method of introducing the tumor-tissue cultures into the host plant involved the thorough grinding of the tissues in a sterile mortar together with a small amount of nutrient-dextrose broth. After an incubation period ranging from a few minutes to 8 hours, the entire tissue suspension was introduced into either sunflower or tomato plants by means of needle punctures. Approximately 100 separate punctures were necessary to use up each tumor suspension. After inoculation the plants were placed in a moist chamber for 18 hours. This was done to prevent the drying out of the droplets of broth and thus permit the multiplication of any bacteria that might be present in the triturated tissues. A total of 32 tumor-tissue cultures were tested, involving more than 3000 separate inoculations. In no case did proliferation of the host tissue result. Galls were invariably produced when bacteria-containing primary-tumor tissue was similarly tested.

The possibility remained, however, that some inhibitory substance might be present in the tumor-tissue cultures that prevented small numbers of bacterial cells from establishing themselves in the host plants. To test this, tumor-tissue cultures were ground up in the usual manner and a few ml. of a dilute suspension of crown-gall bacteria (approximately 1000 organisms per ml.) were added. The tissue suspension together with the bacteria was introduced into tomato and sunflower plants by means of needle punctures. Approximately 50 bacterial cells were inoculated together with a small amount of ground tumor tissue into each puncture. Typical galls were obtained in every instance. It is, therefore, obvious that the ground tumor-tissue cultures did not have any deleterious influence on the gall-stimulating ability of *Phytomonas tumefaciens*.

The presence of some agent in the tumor tissues capable of causing continued abnormal stimulation of the cells, but not itself able to initiate tumor formation, had still to be considered. It is well known that *Phytomonas tumefaciens* may, under certain conditions, become attenuated naturally and that a decrease in virulence also can be brought about by transferring the organism successively through 15 to 20 passages in a medium containing glycine. Both naturally attenuated and glycine-attenuated sister-cell cultures of the A_6 strain of the crown-gall organism were introduced into tomato and sunflower plants, together with thoroughly ground tumor-tissue culture

suspensions. Both of these attenuated cultures were capable in themselves of producing cellular proliferation in the hosts in excess of the normal wound response, but they never formed typical large tumors in their host plants. The plants inoculated with the mixture of a crushed tumor suspension and an attenuated culture showed no more response than did the check plants treated with the attenuated cultures alone. These results show, there-

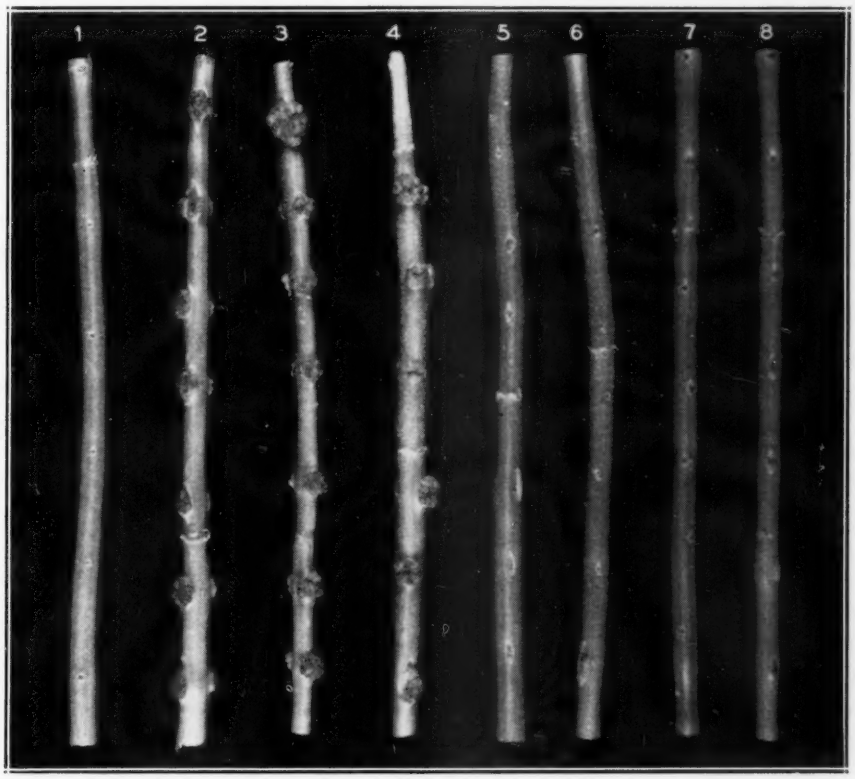

FIG. 2. Summary of plant inoculation studies. Sunflower plants were inoculated by means of the needle-puncture method with: 1, a thoroughly ground tumor-tissue culture; 2, bacteria-containing primary-tumor tissue similarly treated; 3, finely ground tumor-tissue culture plus a dilute suspension of a virulent culture of *Phytomonas tumefaciens;* 4, a virulent culture. Note tumor formation in 2, 3, and 4 where the crown-gall organism was known to be present. 5 and 7 were inoculated, respectively, with a naturally attenuated and a glycine-attenuated culture of the crown-gall organism. In both instances slight proliferation of the host tissue resulted in excess of the normal wound reaction. 6 and 8 were inoculated with the respective attenuated cultures plus a small thoroughly triturated tumor-tissue culture. These results show that there was no demonstrable agent present in the tumor-tissue cultures that could bring about the continued abnormal stimulation of the host cells after tumor formation had been initiated by the attenuated cultures. (Photograph by J. A. Carlile.)

fore, that there was no demonstrable agent present in the tumor-tissue cultures that could bring about the continued abnormal proliferation of the cells after tumor formation had been initiated by attenuated cultures of the crown-gall organism. The results of the plant-inoculation studies are summarized in figure 2.

To ascertain whether some tumefacient agent might be present in or carried by this crown-gall organism, filtrates of virulent cultures were examined. The virulent strains employed were either lysed with specific bacteriophage or were incubated in sterile saline for several weeks to encourage autolysis. The lysed suspensions were filtered through sintered glass bacteriological filters (porosity "65 on 3") or through Berkefeld N candles. The filtrates were introduced into tomato or sunflower plants either alone or in company with a partly attenuated culture of *Phytomonas tumefaciens*. In no case did cellular proliferation result in excess of that found in the check plants. All of these tests indicate that neither filterable bacterial forms nor recognizable viruses were present in tumor-tissue cultures after their prolonged cultivation *in vitro*.

DISCUSSION

The most distinctive characteristic of malignant cells is their independence of the influences that govern the hereditary form of the organism, either animal or plant, in which they occur. All normal cells are subject to a rigid control mechanism when growing *in vivo*.

In work reported elsewhere it has been shown by the writers that the tumor cells in crown gall of plants behave like true malignant animal cells in that they show a greatly increased proliferative power over normal cells, both *in vivo* and *in vitro*, and at the same time retain their tumor-inducing capacity probably indefinitely when grown in tissue cultures or when transplanted in series to suitable host plants.

Speculations as to the origin of malignant cells have been numerous, and these have extended over a period of many years. Two general working hypotheses are now current, intended to explain how malignancy originates. The first of these postulates is that some foreign agent, intimately bound up with the cells, stimulates their growth and multiplication. The second postulate is that normal cells have themselves been modified into new cell types or mutants that are no longer subject to the control mechanisms of the host.

Tumor formation in crown-gall is known to be initiated by a bacterial parasite, *Phytomonas tumefaciens*, and it has generally been assumed that this organism is directly responsible for the continued multiplication of the cells. According to this concept, then, crown-gall tumors would logically fall, like the virus-induced tumors of animals, into the first category listed above, namely, that in which malignancy is attributed to the presence of a foreign agent.

The results of the present investigation have demonstrated, however, that, although *Phytomonas tumefaciens* induces tumor formation, its presence is not always required for the continued rapid proliferation of the cells. This conclusion is based on results of experiments designed not only to eliminate, insofar as the methods now available permit, the possibility of the normal or of aberrant forms of the crown-gall organism being present in the tissues, but also to consider the possibility of a virus that might either be trans-

mitted by or act in association with the bacteria in stimulating cell multiplication. More than 2000 tests designed to demonstrate such agents, bacterial or otherwise, have all given negative results.

The possibility that a growth-promoting chemical might have been introduced into the cells at the time they were first actively stimulated and that this substance, even though continually used up during the multiplication of the cells, might still be present in sufficient quantity to cause them to behave abnormally has not been entirely eliminated. The crown-gall tumor tissue has now been carried in artificial culture for 30 transfers involving a period of more than one year and has increased in theoretical volume more than a hundred million million times; yet, this tissue will have to be carried through at least 13 more generations before the theoretical limits (the equivalent of $\frac{1}{2}$ molecule of H_2O per cell) are reached. However, since the tumor tissues have continued to increase at an *undiminished* rate through 30 generations *in vitro* and have at the same time retained their tumor-inducing capacity undiminished, it seems very unlikely that any such chemical might still be a determining factor in this abnormal behavior.

It seems necessary, therefore, to fall back on the second hypothesis, that a permanent alteration in the cells has taken place. The fact that the tumor cells when cultivated for long periods of time *in vitro* retain their peculiar cultural and cytological characteristics as well as their tumor-inducing capacity, in the absence of any demonstrable stimulating agent, strongly suggests that these are permanently altered cells that reproduce true to type and against which there is no control mechanism in the host. It is clear that, although the original stimulus to abnormal proliferation must have come in some way, either directly or indirectly, from the crown-gall organism, the continuation of this behavior becomes at an early stage quite independent of this original stimulus. It becomes a characteristic of the tumor cells themselves. What the nature of this fundamental change in the character of the cell is we cannot say and it seems needless to speculate at this time. It is believed, however, that the bacteria-free crown-gall tumor cells possess many of the essential characteristics of true malignant animal cells and represent, therefore, a potentially useful material with which to study certain basic principles involved in the etiology of malignancy.

SUMMARY

The bacteriological sterility of tissues derived from secondary crown-gall tumors has been examined.

The results of more than 2000 tests of various kinds are included in the present paper. Viable tumor-tissue cultures have failed to yield cultures of *Phytomonas tumefaciens* in a single instance, when grown on nutrients suitable for the isolation of this organism, when thoroughly crushed and incubated in suitable nutrients, or when cut in thin slices and incubated on media known to favor the growth of this organism.

Special tests suitable for the isolation of small numbers of bacteria or of dormant phases, filterable forms, viruses, or noncultivable forms have likewise consistently yielded negative results.

Implantation of such tumor-tissue cultures in sunflowers and artichoke plants gave rise to typical crown-gall tumors that again failed to yield crown-gall organisms when subjected to the usual tests.

Tumor-tissue cultures, when used as antigens, failed to reveal any crown-gall organisms either by agglutination or complement-fixation tests.

It is believed that the evidence presented provides unequivocal grounds for concluding that the tumor-tissue cultures isolated from secondary tumors are entirely free of *Phytomonas tumefaciens* or of other recognizable tumor-inciting agents.

These bacteria-free crown-gall tumor cells possess many of the essential characteristics of true malignant animal cells, and represent, therefore, a potentially useful material with which to study certain basic principles involved in the etiology of malignancy.

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PERONOSPORA TABACINA ADAM, THE ORGANISM CAUSING BLUE MOLD (DOWNY MILDEW) DISEASE OF TOBACCO

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INTRODUCTION

The fungus causing the blue-mold (downy mildew) disease of tobacco belongs to the Peronosporaceae, which includes a number of important crop-plant pathogens, all of which are obligate parasites. Blue mold is the type of disease that commands attention, and its sudden appearance in the United States as a disease of cultivated tobacco presented students of the fungi with a number of problems. Some of these have concerned:

(1) Present status of identification methods for the tobacco downy mildew and other Peronosporaceae.

(2) Source of the infection in the United States.

(3) Reason for the disappearance of the disease following the 1921 outbreak.

Question 1 involves the broad problem of identification in an important group of plant parasites, and hence being of general interest, will be considered in greatest detail. Questions 2 and 3 are of less general interest, and the writers merely wish to present certain material that leads them to conclusions different in some respects from those recently advanced by Wolf (20) and Stevens and Ayres (17).

THE IDENTIFICATION OF PERONOSPORA TABACINA BY CONIDIOPHORE AND CONIDIA MEASUREMENTS

Students of the Peronosporaceae have used extensively length and breadth measurements of conidia in describing and identifying species. Most descriptions are based on material collected on a single host plant, which, in the case of *Peronospora tabacina* Adam, was *Nicotiana tabacum*. However, *P. tabacina* attacks many other species of *Nicotiana*, and a question that naturally arises is whether spores collected on other host species would be comparable in size with those collected on *N. tabacum*. To obtain definite information, the following experiment was conducted.

Thirty collections of blue-mold leaf material were gathered from 12 different species of *Nicotiana*, all grown in a single greenhouse during the period October, 1940, to April, 1941. There was no outside source of inoculum during this time, so all collections may be assumed to be the same fungus, more particularly because separate strains of the blue-mold organism are unknown. Each different collection from the same species

[1] The writers acknowledge the efficient assistance of Alice J. Watson, formerly of the Division of Mycology and Disease Survey, Bureau of Plant Industry, U. S. Department of Agriculture.

of *Nicotiana* (Table 1) was taken at a different time and from a different crop of plants.

To provide adequate "grand mean" values with which to compare the previous collections, 40 lots of blue-mold-infected leaves were obtained from *Nicotiana tabacum*. These collections were made over the same period, October, 1940, to April, 1941. All collections were preserved alike, and spore measurements were made by one person for reasons set forth by Harter (13). For each collection, 10 measurements were made of conidiophore length, and 25 each of conidia length and breadth. All were searched carefully for oospores, and, where found, 10 measurements were taken of the outside diameter. The objective was to determine whether by spore measurements made under the most favorable conditions it would be possible to prove that a downy mildew collection on *N. attenuata*, for example, was the same as the downy mildew of cultivated tobacco. As a test of whether the spore sizes recorded did or did not show significant differences, the writers have subjected the entire mass of data to statistical analysis. The formula for measuring significance was

$$t \sqrt{\frac{\text{Error } M^2(N_1 + N_2)}{N_1 \times N_2}}$$

Analysis of the data shows that despite the fact that conidiophore length varied considerably within collections, and hence a rather large difference was required for significance, 23 out of the 30 collections differed significantly in size from the grand mean, and all but one of the differences were significant at the 1 per cent level. Thus, the species collection measurements tended to be different from the *Nicotiana tabacum* grand mean, and hence the measurements did not provide a positive basis for identification. Furthermore, the differences were not consistent. Thus one collection on *N. stocktoni* had longer conidiophores and one, shorter conidiophores than the grand mean.

Conidia length and breadth has been the generally preferred method for identifying *Peronospora* species. However, considering length and breadth, 25 of the 30 collections were significantly different in one or both of these dimensions; and, again, most of the differences were significant at the 1 per cent level. Hence, it must be concluded that the conidia dimensions of the *Nicotiana* species test collections tend to differ from the *N. tabacum* grand mean.

Further analysis of the data in table 1 has shown (a) that the differences between the 40 mean values for *Nicotiana tabacum* collections were highly significant, and (b) that the variability was still greater between the 30 mean values for the *Nicotiana* collections. Hence the production of conidia on different host plants increased the amount of size variability. Variation *within samples* was substantially the same regardless of host plants.

It has been suggested (22) that a reason for the variability in pub-

TABLE 1.—*Measurements of Peronospora tabacina conidiophores and conidia*

TABLE 1.—*Measurements of Peronospora tabacina conidiophores and conidia*

Collection number	Host plant *Nicotiana*	Conidiophores—length		Conidia—length		Conidia—breadth	
		Range μ	Mean μ	Range μ	Mean μ	Range μ	Mean μ
21	*attenuata*	321–465	380.1[b]	16–22	18.44	13–19	15.42[b]
31	"	225–366	300.1[c]	15–22	18.56	11–17	15.04
14	*benthamiana*	264–420	346.5[c]	13–21	18.56	12–19	15.00
8	"	180–300	246.9[c]	15–20	17.24[c]	12–17	14.84
9	"	225–330	277.2[c]	14–21	18.16[b]	13–18	15.08
13	*quadrivalvis*[a]	324–540	432.3	14–21	16.96[c]	12–16	13.80[c]
33	*caesia*	480–780	632.1[c]	16–24	18.32[b]	11–17	13.04[c]
15	*glauca*	312–511	412.8	15–21	17.28[c]	12–17	14.56
23	"	450–762	584.1[c]	15–22	17.88[c]	12–16	13.88[c]
30	"	510–954	721.8[c]	16–21	18.04[c]	12–18	14.32
32	"	363–615	471.6	15–23	18.52	12–16	13.64[c]
38	"	409–996	624.1[c]	15–21	17.84[c]	12–15	13.92[c]
51	*gossei*	270–348	303.9[c]	15–22	18.36	12–17	14.56
22	"	276–423	337.8[c]	16–22	18.28[b]	12–19	14.52
5	*nesophila*	315–420	364.4[c]	12–24	16.96[c]	12–18	13.72[c]
11	"	213–309	249.9[c]	13–21	17.60[c]	12–17	14.60
24	"	306–705	546.6[c]	15–21	17.92[c]	12–16	14.36
28	"	249–360	309.1[c]	16–21	18.36	11–17	15.12
39	"	240–336	294.6[c]	16–22	18.60	13–19	15.40[b]
42	"	252–354	310.5[c]	15–20	17.80[c]	13–16	15.12
10	*repanda*	345–600	463.5	15–24	19.52	13–19	15.36
72	"	414–621	514.8[c]	15–23	18.64	11–19	15.90[c]
34	"	405–681	572.7[c]	16–21	18.20[b]	12–15	14.32
37	*stocktoni*	336–477	415.8	13–19	16.92[c]	12–16	13.92[c]
26	"	279–330	307.3[c]	16–21	18.96	13–19	15.72[c]
41	"	309–783	549.0[c]	15–20	17.88[c]	10–16	13.64[c]
16	*sylvestris*	405–825	589.5[c]	15–24	18.08[c]	12–16	14.08[c]
29	"	321–456	393.3	15–23	19.20	11–18	14.16[b]
17	*tomentosa*	270–540	366.5[c]	14–21	17.12[c]	12–16	13.32[c]
27	*wigandioides*	318–570	456.6	15–21	18.08[c]	12–17	14.04[c]
	N. tabacum grand mean derived from 40 collections	330.4–552.48	433.9	16.0–23.33	19.13	12.58–17.75	14.83

Significant difference between mean of any sample and grand mean of 40 samples of *N. tabacum* (calculation based on individual values from all 70 collections):

at 5 per cent level	50.4	0.78	0.54
at 1 per cent level	66.3	1.02	0.71

[a] Received as *N. bigelovii* var. *quadrivalvis*.

[b] Significantly higher or lower than average of 40 samples of *N. tabacum* at 5 per cent level.

[c] Significantly higher or lower than average of 40 samples of *N. tabacum* at 1 per cent level.

TABLE 2.—*Measurements of Peronospora tabacina conidia reported by previous investigators*

Report by	Conidia			
	Length		Breadth	
	Range	Mean	Range	Mean
	μ	μ	μ	μ
(1) Adam	16–29	22	13–19	17
(2) Angell and Hill	.....	18–28	.....	14–17
(3) Armstrong and Albert	.....	17.9–19.5	.....	13.1–15.4
(10) Godoy and Coste[a]	15–24.5	21	12–16	14.5
(21) Wolf *et al.*[a]	15–28	.....	12–18	.....
(22) Wolf *et al.*	10.5–24	18.4	10.5–22	15

[a] The name *P. nicotianae* was used.

lished reports on *Peronospora tabacina* spore size might be that too few measurements were made. Thus if a person made enough measurements they would reach a true value. However, table 1 shows that with means based on only 25 measurements the difference required for significance was only about 5 per cent. This is very low, and proves that merely increasing the number of measurement within collections would not solve the problem. If, under the relatively uniform conditions provided, conidia measurement failed as a means for *P. tabacina* identification, what would be expected under more variable conditions?

To answer this question, the data reported by previous investigators are given in table 2.

TABLE 3.—*Conidia size of Peronospora species as reported by Berlese and Gäumann*

Peronospora Group	Peronospora Species	Host plant Genus	Conidia length		Conidia breadth	
			Range[a]	Mean[b]	Range[a]	Mean[b]
			μ	μ	μ	μ
Leiothecae		*Dipsacus* *Knautia* *Scabiosa*				
	P. violaceae	*Succisa*	30–47	35	20–26	19
	P. phyteumatis	*Phyteuma*	20–26	21	14–17	14
	P. urticae	*Urtica*	20–27	30	18–22	22
	P. valerianellae	*Valerianella* *Papaver*	23–28	25	16–20	19
	P. arborescens	*Meconopsis*	20–24	16	16–20	15
	P. grisea	*Veronica*	22–27	24	16–20	16
	P. chrysosplenii	*Chrysosplenium*	20–25	24	16–19	20
	P. lamii	*Lamium*	18–24	27	15–20	21
	P. candida	*Anagallis*	22–26	19	16–20	16
	P. antirrhini	*Antirrhinum*	25–27	25	15–17	21
	P. ficariae	*Ranunculus*	20–29	27	15–20	22
Calothecae						
	P. calotheca	*Asperula*	20–23	26	12–17	16
	P. myosotidis	*Myosotis*	16–24	21	12–15	16

[a] Conidia ranges from Berlese.
[b] Conidia means from Gäumann.

The data in table 2 show a maximum range in conidia length of 10.5 to 29 μ with mean values from 17.9 to 28 μ, and a maximum range in breadth from 10.5 to 22 μ with mean from 13.1 to 17 μ. The spread is very much greater than in table 1, which could be attributed to more variable environmental conditions, and the fact that the material was handled differently and the measurements made by different persons. Considering the range of values in both tables 1 and 2, it appears reasonably certain that *Peronospora tabacina* could not be definitely identified by conidia measurements, and it is of interest to know whether the situation with *P. tabacina* is the exception or the rule.

To test this point, 13 species were selected at random from the classical monographs of Berlese (5) and Gäumann (9), the former giving conidia size in terms of range, the latter in terms of the mean. Table 3 lists the two sets of measurements. It will be noted that in 10 of the 13 species Gäumann's

TABLE 4.—*Measurements of Peronospora tabacina oospores*

Collection No.	Diameter	
	Range	Mean
	μ	μ
43	27–39	32.9
44	30–38	34.5
45	33–40	37.5
46	30–39	34.2
48	33–45	39.9
50	27–34	30.6
51	30–36	32.4
65	24–31	27.8
70	27–36	32.7

Significant difference between any 2 means at 5 per cent level ..... 2.9 μ
Significant difference between any 2 means at 1 per cent level ..... 3.9 μ

measurements lie completely outside the range given by Berlese. For only 3 species, do the two sets of measurements correspond. This appears to be good evidence that Peronospora conidia measurements quite generally tend to differ, and a large amount of other corroboratory data could be cited.

OOSPORES AS AN AID TO THE IDENTIFICATION OF P. TABACINA

Out of the 70 collections studied, oospores were found in 9, and all of these were *Nicotiana tabacum*. Thus, it is evident at the start that under ordinary circumstances comparatively few collections may be expected to show oospores. Angell and Hill (2) stated that of hundreds of collections made in Australia, only one had oospores. The measurements of the oospores in each of our 9 collections are given in table 4.

Table 4 figures indicate a great variability in the size of *Peronospora tabacina* oospores. Computations show that many of the differences between mean values were highly significant. Thus, collections 48 and 50, made simultaneously in the same area, have a mean difference of 9.3 μ, and the

difference required for significance at the 1 per cent level was only 3.9 μ. Collection 48 was the largest, and collection 65 the smallest, and as between these two, 48 was 37 per cent greater in minimum diameter and 45 per cent greater in maximum diameter. Furthermore, the ranges of oospore size of different collections may not even overlap. Thus, the range of collection 65 was 24 to 31 μ, and for collection 48, 33 to 45 μ. Despite the variability of the figures in table 4, however, it might reasonably be expected that they do not represent a maximum of variability, because they were all collected in one season in one general area and handled in all respects alike. Table 5 lists the oospore measurements for *P. tabacina* that have been previously published.

The variability represented by the *Peronospora tabacina* oospore measurements reported in the literature and collected in table 5 is rather amazing. The range as reported by Wolf alone is 24 to 75 μ, and in Australia the combined measurements of Adam and Angell and Hill provide a range of

TABLE 5.—*Measurements of Peronospora tabacina oospores reported by previous investigators*

Report by	Diameter	
	Range	Mean
	μ	μ
(1) Adam	35–60	46
(2) Angell and Hill	28–50	.....
(7) Clayton and Stevenson	.....	32–40
(21) Wolf *et al.*, 1934[a]	45–75	.....
(22) Wolf *et al.*, 1936	24–43	32

[a] The name *Peronospora nicotianae* was used.

28 to 60 μ. All the oospore measurements for the Peronosporaceae as listed by Gäumann and Berlese provide a range for the entire genus of only 22 to 60 μ, so that it is evident that for *P. tabacina* the reported dimensions as given by highly responsible workers cover somewhat more than the accepted range for the genus. Furthermore, the minimum mean size—32 μ—as reported by Clayton and Stevenson (7), and Wolf *et al.* (22) is only about 70 per cent of the general mean of 46 μ reported by Adam (1) in his original description of *P. tabacina*. It seems apparent that *P. tabacina* oospores may be considered as not distinctive in appearance and extremely variable in size.

THE STATUS OF P. NICOTIANAE

In 1898, Spegazzini (16) described as *Peronospora nicotianae* a downy mildew that he collected on a native *Nicotiana* in Argentina. This species has been considered distinct from *P. tabacina* by Adam (1) and others on the basis of an apparent difference in size of conidiophores and conidia. These supposed differences in size do not exist, however, as the writers have pointed out previously (7), but there still remain two features of the Spegazzini description that do not fit the blue-mold fungus found in the

United States. First, Spegazzini stated that his conidia germinated indirectly, to form zoospores, and second he described as oospores bodies, ranging in size from 50 to 80 μ, that are very different in appearance from the oospores found associated with the blue-mold fungus in Australia and the United States.

Prior to 1935, no Nicotiana downy mildew had been reported from South America since the Spegazzini report of 1898. Within the past 5 years, however, Wolf (20) has reported receiving *N. tabacum* material with typical

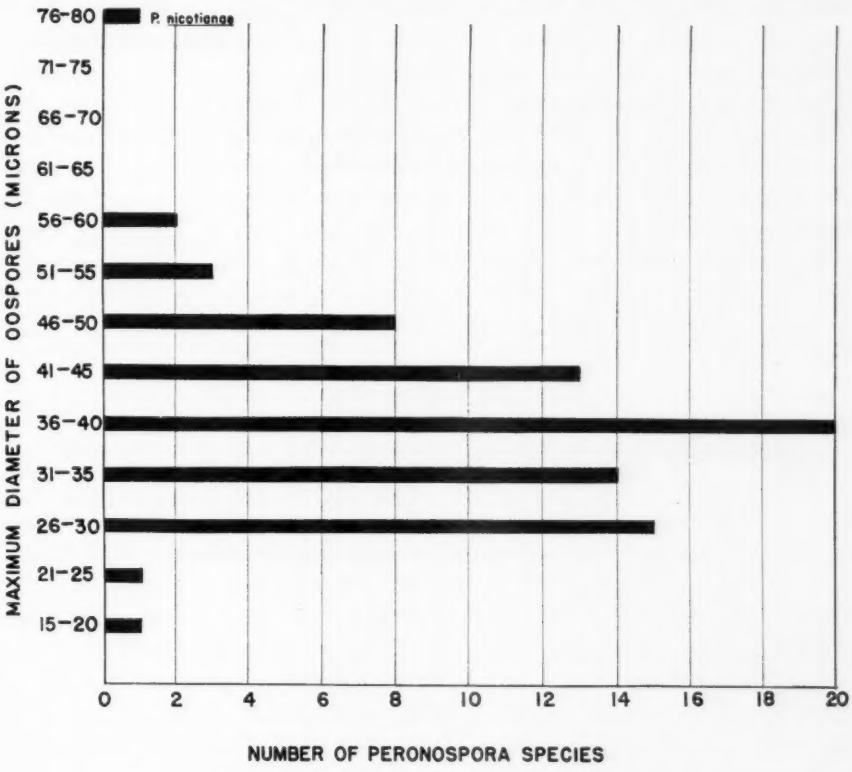

FIG. 1. The range of oospore size in the genus *Peronospora*.

Peronospora tabacina oospores from Rio Grande do Sul, that portion of Brazil nearest Argentina; and the writers have received similar material from the same area, which did not, however, show oospores. Still more recently, Godoy and Coste (10) reported a downy mildew of cultivated tobacco in Argentina. Thus, it is now established that a downy mildew, similar to that in the United States and Australia, is a serious disease of cultivated tobacco in the South Brazil-North Argentina area. Godoy and Coste searched for the oospores reported by Spegazzini, but reported their failure to find any. They recorded their organism as *P. nicotianae* on the grounds that the conidia and conidiophore dimensions did not agree with *P. tabacina*. As table 2 shows, they really agree very well. The situation is,

consequently, that, since the Spegazzini report of 1898, no one has found the bodies he believed to be the oospores of his *Peronospora*. It has been suggested that these bodies appear to be typical *Peronospora* oospores. However, as figure 1 shows, a maximum size of 80 is completely outside the normal oospore size range of the genus; in fact, so far as the writers are aware, no one, excepting Wolf *et al.* (21), has reported *Peronospora* oospores in excess of 60 μ in diameter. Secondly, as *Peronospora* oospores they would fall in the group Calothecae, while *P. tabacina* is in the Leiothecae. Search through Gäumann shows that while Gäumann lists as many as 7 separate *Peronospora* species in one genus (*Ranunculus*), in no case does he report a single host genus attacked by *Peronospora* species that falls in the 2 separate groups. Thus, in the instance referred to, all 7 *Peronospora* species, parasitic on *Ranunculus*, were members of the Leiothecae. It appears, consequently: (1) that a fungus fitting the *P. nicotianae* description has not been observed since its original description in 1898; (2) that a fungus answering the *P. tabacina* description is very definitely present in the area from which *P. nicotianae* was reported; and (3) that the supposed oospores on which *P. nicotianae* is largely based are completely outside the known oospore size range for the Peronosporaceae. In view of these considerations, the existence of *P. nicotianae*, as described, is so seriously in doubt that it can be omitted from further consideration.

THE STATUS OF PERONOSPORA TABACINA AS AN ENDEMIC OR INTRODUCED FUNGUS IN NORTH AND SOUTH AMERICA

Many years ago it was suggested by McAlpine (15) that *Peronospora tabacina* was probably endemic to Australia, and this view is now quite generally accepted. With respect to North America, Stevens and Ayres (17) have recently suggested that the evidence for endemism is very scant, and Wolf (20) regards *P. tabacina* as an introduced species in South America. A problem of this sort is not easy to settle conclusively, but the writers believe there is strong evidence in favor of the view that the fungus is endemic in North and South America, as well. In the first place, *P. tabacina* is an obligate parasite, dependent for its existence on the presence of suitable food plants, *i.e.*, members of the genus *Nicotiana*. It is very different, thus, from many fungi that can apparently exist for long periods as saprophytes, or which attack species scattered through many genera. It is true that *P. tabacina* attacks peppers and, to a very slight extent, tomatoes and egg plant (3). However, so far as known, these other species are only affected in areas where the primary infection could be derived from *Nicotiana*. Godfrey (11) recently collected downy mildew-affected pepper plants in Texas, and, on subsequent search, found heavily infected *Nicotiana repanda* in the near vicinity. The dependence of *P. tabacina* on *Nicotiana* host plants for food would limit endemism to areas having a native *Nicotiana* flora. These areas are North and South America and Australia. *P. tabacina* is, of course, a low-temperature organism, which would limit its occurrence

to temperate regions. The temperate-zone regions with native *Nicotianas* are (1) south Australia, (2) western United States, and (3) south Brazil and north Argentina. The early mycologists collected a *Peronospora* on the native *Nicotianas* in all these areas.

The first records of a tobacco downy mildew in the United States involved *Nicotiana glauca* and *N. bigelovii*, which table 1 shows are good host plants for *Peronospora tabacina*. It is interesting to note that tobacco growers in the United States were not troubled by a downy mildew from 1620 to 1921, due, apparently, to the fortunate circumstance that tobacco culture was confined to the East, and no native *Nicotianas* occur east of the Mississippi River. How the gap was finally bridged remains speculation.

It may be mentioned here that the tobacco cultivated by the Indians in the East prior to the arrival of white settlers was not *N. tabacum* but *N. rustica*, which is quite resistant to blue mold and, accordingly, does not enter into the present problem.

The view that *P. tabacina* is endemic to Australia, and was introduced into North and South America, the only other areas with a native *Nicotiana* flora, while such regions as New Zealand, South Africa, Europe, and Asia have all remained free from such an introduction, does not appear to be very plausible.

THE DISAPPEARANCE OF TOBACCO BLUE MOLD IN 1921

The first appearance of this disease in 1921 caused much concern, and its failure to reappear the following year was a surprise. Stevens and Ayres (17) have recently discussed the theory that this failure to reappear might have been due to climatic conditions. After a study of available records, they, however, concluded that it would not appear that the presence of the disease was correlated with the weather. The writers have examined leaf material collected in 1921 and find no oospores present. Wolf *et al.* (22) have very clearly shown the dependence of the organism on oospores for overwintering. Hence, the suggestion is made that the fungus, in 1921, failed to form oospores and so died out. The area affected in 1921 was very limited. In recent years large areas have been repeatedly observed where oospores could not be found, and there was no indication of old-bed overwintering in these regions the following spring. At present, the area involved each year is so extensive that the chance of oospores not being formed is small. It is quite possible that oospores may live over several years. Certainly, the lack of success experienced by workers in attempting to germinate them would suggest such a possibility.

DISCUSSION AND CONCLUSIONS

A study has been made of the value of conidiophore, conidia, and oospore measurements in the identification of *Peronospora tabacina*. Conidiophore length has been found to be both extremely variable and inconsistent, so that it has little or no taxonomic value. Results from conidia

measurements were more consistent, thus corroborating to this extent the conclusion of Gäumann that such measurements were the most suited to taxonomic purposes. However, it was conclusively proved that mean values for different collections of conidia tend to differ. Thus, when 30 collections were compared with grand mean values set up with the utmost care, the great majority of the 30 collection measurements were significantly different from the grand means. Consequently, the effort to establish a single mean value for the size of *P. tabacina* conidia does not have a sound basis. This view is supported by the findings of Angell and Hill (2), who reported that the size of conidia produced at different times of the year varied greatly, and Armstrong and Sumner (4), who found smaller but very significant differences for *P. tabacina* spores produced on different host plants. Analysis of the data in table 1 also showed that the variability in the mean values obtained from collections made from different *Nicotiana* species was much greater than where collections were made from a single host plant—*Nicotiana tabacum*. That variability in Peronospora conidia size is not limited to *P. tabacina* is strongly suggested by table 3, in which the spore ranges of Berlese and the means of Gäumann are compared for 13 species. It is apparent there that the correspondence between the 2 sets of values is generally poor. Also, workers have sought for years to decide whether *Peronospora hyoscyami* was the proper name to use for the tobacco organism as suggested by Farlow (8). On the basis of conidia size, it is possible to prove (1) they are the same size or (2) *P. hyoscyami* is larger, depending entirely on which of the various published measurements are used.

Taking *Peronospora tabacina* mean values for conidia size as given in tables 1 and 2, it is evident that mean length can range from 17 to 28 μ and mean breadth from 13 to 17 μ. However, such a range of mean values includes many other species of *Peronospora*, and hence conidia measurements cannot be relied upon definitely to identify *P. tabacina*. On the other hand, mean values above or below this range would be a strong indication that the fungus in question was not *P. tabacina*. Gäumann used oospore type to differentiate the genus *Peronospora* into 2 main groups—Leiothecae and Calothecae—but he concluded that the oospores were too similar in appearance, and too variable in size to be of help in species identification. Stevens and Ayres (17) recently have suggested that a definite identification of *P. tabacina* could be made only if the oospores were present. *P. tabacina* oospores however, have nothing to separate them from *P. hyoscyami* and dozens of other members of the Leiothecae group. They have an irregularly wrinkled wall without definite pattern. In table 4 the mean diameter values for 9 collections range from 27.8 to 39.9 μ—a variation of 43 per cent. Most workers have listed oospores size as ranges of individual values. Table 4 shows such a range for one collection to be 24 to 31 μ, and for another 33 to 45 μ. In table 5 the reports of *P. tabacina* oospore size by other investigators show a range 24 to 75 μ, while the range for the genus *Peronospora*, as given

by both Berlese and Gäumann, is only 22 to 60 μ. Thus, if anyone were to discover oospores associated with a downy mildew on *Nicotiana* that had measurements outside the published range for *P. tabacina*, they would be justified in concluding that the bodies under observation were probably not *Peronospora* oospores. From the diagnostic viewpoint, the great potential value of oospores would appear to be in providing material for pathogenicity studies. Up to the present, however, most workers have been unable to obtain germination.

The conclusion derived from the preceding is that *Peronospora tabacina* cannot be definitely identified on the basis of size or appearance of its conidiophores, conidia, or oospores. Furthermore, it is indicated that the same may hold true quite generally for the Peronosporaceae. However, while these fungi appear to be not sharply separated morphologically, they are very highly specialized with respect to parasitism. *P. tabacina* is practically limited to the genus *Nicotiana*. The decisive value of pathogenicity in species differentiation is well illustrated by the *P. hyoscyami* situation. The tobacco fungus went by this name from the time that Farlow (8) collected a downy mildew on *N. glauca* in California, which had conidia about the same size as *P. hyoscyami*. There was doubt as to whether the tobacco fungus was the same, but repeated sets of conidia measurements and the discovery of oospores of both species (1, 5) failed to settle the question. However, this problem was definitely settled when it was found quite recently (2) that the tobacco organism did not attack *Hyoscyamus niger*.

The Peronosporaceae as classified by Gäumann contain numerous separate species that are differentiated solely on the basis of conidia length and breadth. The writers do not believe that conidia size is sufficiently stable to make such differentiation valid, particularly in view of the fact that size values assigned to many species were based on few, and in some cases apparently a single collection. It appears that the true species situation can be settled only by extensive controlled cross-inoculation work. At present there is very little information of this sort available. In this connection, the recent findings of Hoerner (14) are suggestive. He demonstrated by inoculation experiments that the supposedly separate *Pseudoperonospora* species—*P. cannabina*, *P. celtidis*, *P. urticae*, and *P. humuli*—may be merely physiologic races of a single species.

A fairly complete study of the pathogenicity of *Peronospora tabacina* with respect to the various *Nicotiana* species has been completed and will be published. This should provide a definite basis for as thorough identification of *P. tabacina* as may be desired, and it should also make it possible to determine whether specialized races of the organism exist in the different regions affected.

At present there appear to be no grounds for belief that more than one *Peronospora* occurs on *Nicotiana*. Spegazzini's *P. nicotianae* has not been observed since it was described, and the supposed oospore bodies on which it is chiefly based are much too large (Fig. 1) to be those of *Peronospora*. If

P. tabacina is the only *Nicotiana* downy mildew, then the need for assuming that it has been introduced into South America disappears. The writers believe the evidence strongly favors the view that the tobacco fungus is native to North and South America and Australia.

It is suggested that a likely explanation of why blue mold disappeared after the 1921 outbreak in the Georgia-Florida area of the United States is that the fungus failed to produce oospores.

SUMMARY

In considering the identification of *Peronospora tabacina*, it was found that mean values for length and breadth of conidia and length of conidiophores tend to differ significantly, and the true situation is better represented by a range of mean values, rather than a single mean.

Size variability was greater with collections made from different host plant species than with collections made from a single host plant species.

The range of means indicated for *Peronospora tabacina* conidia is 17 to 28 μ long by 13 to 17 μ broad. These ranges are too great, however, to permit definite identification by measurements.

The variations in oospore size are such that measurements could have little or no taxonomic value other than to indicate that the bodies in question were within or without the size range for the genus, which is approximately 20 to 60 μ.

Neither conidiophores, conidia, nor oospores have distinctive morphological characters that could aid species determination.

Evidence is offered to show that the condition prevalent with respect to the tobacco organism is representative of the general situation in the genus *Peronospora*, and that spore measurements generally have not provided a sound basis for species identification.

The only definite basis available appears to be pathogenicity, and the fungus causing blue mold of tobacco is practically limited in its parasitism to the genus *Nicotiana*.

There appears no reason to believe that more than one *Peronospora* attacking *Nicotiana* exists. The name accepted for this organism is *P. tabacina* Adam.

This fungus is believed native to all temperate zone regions having a native *Nicotiana* flora, *i.e.*, portions of North and South America and Australia.

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CERCOSPORA BLIGHT OF CARROT[1]

H. REX THOMAS[2]

(Accepted for publication May 30, 1942)

This study originated as a field investigation of carrot blight at Santa Maria, California. It is concerned primarily with the physiology of *Cercospora carotae* (Pass.) Solheim and with the host-pathogen relationships and epidemiology of Cercospora blight.

PLANTS ATTACKED

Cercospora carotae is known to attack both wild and cultivated carrots (*Daucus carota* L., *Daucus carota* L. var. *sativa* DC.). Infection was obtained on *D. hispanicus* Gouan, *D. maritimus* Lam., *D. pulcherrimus* Koch ex DC., *D. maximus* Desf., *D. gingidium* L., *D. pusillus* Mich., and 107 carrot varieties and selections tested in the field for resistance to Cercospora blight. Although the species *D. pulcherrimus*, *D. pusillus*, *D. gingidium*, and *D. maximus* were less severely blighted, no important degree of resistance suitable for use in a breeding program was found.

SYMPTOMS

Cercospora blight may attack any part of the leaf, but primary lesions usually are located along the edge of the leaflets, causing a lateral curling (Fig. 1, A). Such lesions are elongate, while those that are not marginal tend to be circular. A diffuse chlorotic border may surround the necrotic portion of the spot. The lesion first appears as a pin-point chlorotic area, which enlarges and soon develops a necrotic center. These small spots enlarge and coalesce until the whole leaflet is killed. During humid weather, the lower surface of the lesion usually appears light gray or silvery because of the characteristic mass of hyaline conidia. This is one of the most reliable macroscopic means of differentiating between Cercospora and Macrosporium blights. *Cercospora carotae* may sporulate abundantly on the surface of the blade or petiole before the host tissue is killed.

Linear blackish-gray lesions develop, which may cover the entire petiole of the older leaves (Fig. 1, B). These lesions, like those on the blade, develop a light gray or silvery cast when conidia are produced. Eventually the petiole may be girdled and the leaf killed.

[1] Joint contribution from the Division of Plant Pathology, University of California, Berkeley, and the Department of Botany, Purdue University Agricultural Experiment Station, Lafayette, Indiana, based on a thesis submitted to the Graduate School of the University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Journal Paper No. 13, of the Purdue University Agricultural Experiment Station.

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STOMATAL PENETRATION

Stomatal penetration was studied both in stained sections of leaf tissue and in material imbedded in paraffin. Considerable difficulty was experienced in observing penetration, as less than 1 per cent of the germ tubes penetrated. All attempts to increase materially the percentage of invasions by exposing the plants to light, darkness, and various temperatures during the incubation were unsuccessful. Examination of several thousand germinating conidia revealed no evidence of direct penetration of the epidermis.

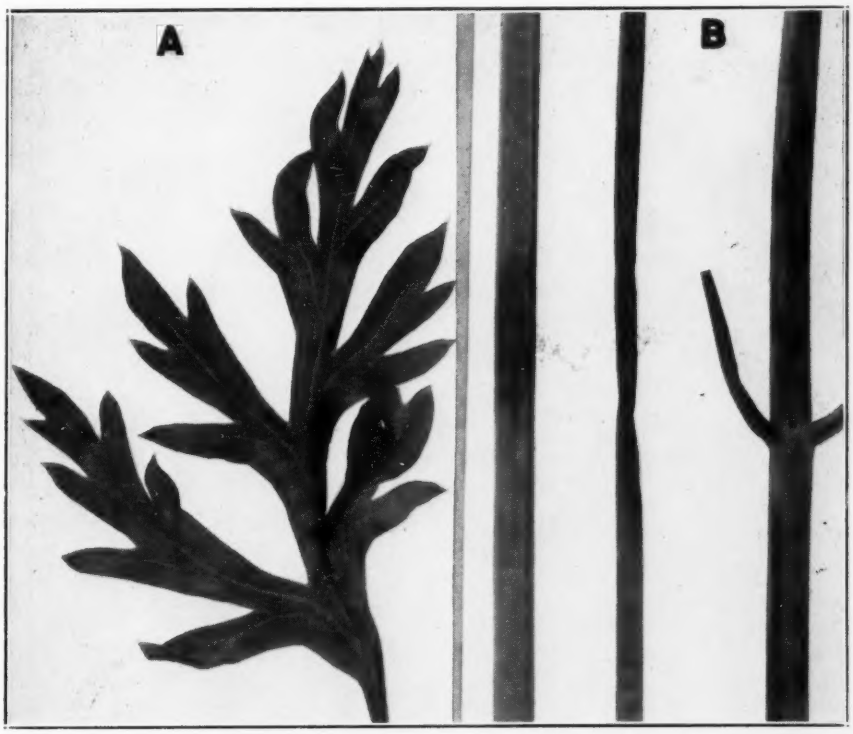

FIG. 1. A. *Cercospora carotae* lesion on portion of a carrot leaf. Note elongate marginal lesion and slight lateral curling of leaflet. B. *C. carotae* lesions on the petiole. Note linear shape of the lesion and silvery cast caused by the mass of conidia on the surface.

Some stomatal penetration occurred within 36 hours, but it was found most abundantly in material fixed after 48 to 72 hours. Stomata apparently had no attractive influence on germ tubes, as many of them grew across stomata without penetrating. Germ tubes appeared to grow at random until they accidentally came in contact with a stoma. No distortion of the somatal opening was caused during penetration, and no appresoria were found.

Entry through stomata is a phenomenon exhibited by other Cercospora species, such as *C. beticola* Sacc. (9) and *C. apii* Fresen (4), although wound infection is reported for *C. fabae* Fautrey (15).

DEVELOPMENT OF THE FUNGUS IN THE HOST TISSUE

The development of the mycelium inside the host was studied by examination of paraffin sections of leaves made 48, 72, 96, and 130 hours after inoculation with conidia. The penetrating hypha often enlarges after it has entered the stoma, plugging the stomatal cavity with globoid, thick-walled cells. The advancing hyphae usually invaded the mesophyll before advancing far laterally in the epidermis. Exceptions to this procedure were occasionally noticed when complete collapse of several epidermal cells would be found with no evidence of the fungus in the underlying tissues. Usually, such groups of cells were close to a point of stomatal infection. The advancing hyphae are intercellular at first, but intracellular invasion occurs promptly. Groups of collenchyma cells on the ridges of the petiole acted as a normal barrier. Very often mycelium would be abundant on both sides of one of these groups of collenchyma cells, but no hyphae were found in this tissue (Fig. 2). The fungus did not penetrate the petioles more than 5 or 6 cells in depth, growing chiefly in a lateral direction. In the leaf blade the fungus often completely permeated the mesophyll within 5 days.

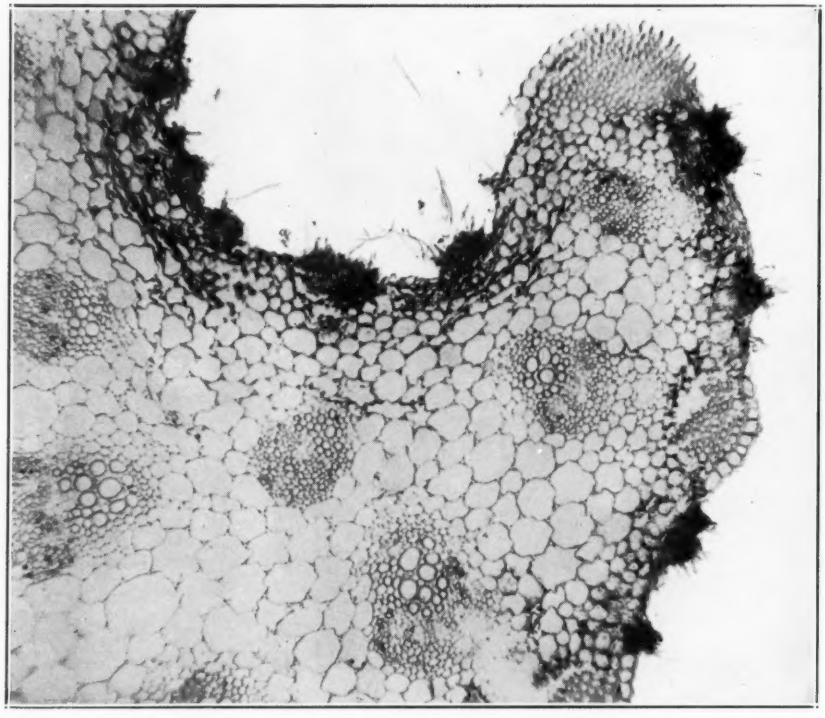

FIG. 2. Cross section of carrot petiole. Note that the fungus has not penetrated deeply and that conidiophore masses are found between groups of collenchyma. ×350.

SPORULATION

After the advancing hyphae have invaded to an extent of several cells,

vertically and laterally, the intertwined mass of substomatal hyphal cells sends up a tuft of hyphae that disrupts the stomatal opening and produces a fascicle of conidiophores 60 to 122 μ wide. On most of the petiole lesions a very compact fascicle of conidiophores was found as contrasted to the smaller and less compact conidiophore tufts found on both surfaces of the leaves. Occasionally, on the leaf, a single conidiophore was developed from a small group of cells in the stomatal cavity soon after infection. Each conidiophore produces several conidia in succession and 2 or 3 scars often are found near the tip of the conidiophore. The conidiophores, 2 to 3 μ in diameter, usually were not enlarged at the base. The conidia were cylindrical and hyaline to slightly colored. Among 100 conidia measured, there was a range in length between 40 and 110 μ with a mean of 95 ± 13.5 and a range in width between 2 and 2.5 μ with a mean of 2.2 ± 0.1. The number of septa varied from 1 to 6.

CULTURAL CHARACTERS

Isolation of the fungus may be easily accomplished by making dilution platings of conidia on potato-dextrose agar. The conidia germinate within 12 hours on agar. The terminal cells usually germinate apically, the lower cells laterally. Very erratic germination occurred in hanging-drop preparations.

The fungus grows very slowly in culture, and it is 4 or 5 days before the growth from conidia on agar is macroscopically visible. The germ tubes and mycelium produced on agar are composed of short, globoid cells as contrasted to the long, narrow cells produced by conidia germinating in water. On agar a dark, dense, compact colony is formed on which conidia are usually present by the time the colony is visible. Cultures grown on artificial media for several months frequently lost their ability to produce numerous conidia. However, if a passage was made through carrots, such cultures produced abundant conidia.

The amount of growth and sporulation of the fungus on 8 kinds of media, adjusted to pH 6.2, was estimated in tube cultures kept at 22° C. for 3 weeks. For a more accurate measure, the rate of growth of hyphae from single conidia for 11 days after germination was determined microscopically. The most vigorous growth occurred on potato dextrose, but there also was good growth on pea, carrot-root, and prune agar. The poorest growth was made on malt and corn-meal agar. More conidia were produced on potato-dextrose, carrot, and carrot-leaf agar, than on pea, corn-meal, malt, and prune agar.

EFFECT OF TEMPERATURE, HUMIDITY, AND pH ON THE GROWTH OF THE FUNGUS

The temperature relationships of the fungus were determined by estimating the amount of growth produced on potato-dextrose agar slants at temperatures of 3-degree intervals between 7° and 37° C. For more accurate measurements, the average maximum radial growth on agar plates of

hyphae from 10 conidia, after 11 days' exposure to the different temperatures, was microscopically measured. Most rapid growth occurred between 19° and 28° C., and there was almost no growth at 7° or 37° C.

The effect of temperature on the percentage germination of conidia was determined in a water suspension on glass slides covered with a thin layer of agar held for 24 hours in Petri-dish moist chambers at different temperatures. The maximum germination occurred between 16° and 28° C. and the least at 7° or 37° C.

The effect of temperature on sporulation was determined by placing 3 carrot petioles with young lesions at temperatures of 3-degree intervals between 7° and 37° C. After 24 hours it was found that abundant sporulation occurred between 13° and 28° C. No conidia were formed at 7° or 37° C.

Drops of a conidial suspension were placed on agar-coated slides and allowed to dry in air-tight, glass-stoppered bottles in which humidities ranging from 21.5 to 100 per cent were maintained by means of different concentrations of sulphuric acid. Although a few conidia germinated at 93.2 per cent humidity, no appreciable number germinated at less than 99 per cent. At humidities so close to 100 there is always a chance that condensation may have occurred. It was concluded that very little germination occurs unless a film of moisture is present. The conidia in the lower humidities were not injured because they always germinated satisfactorily when removed and exposed to moist conditions.

On agar slants the maximum amount of growth was made between pH 5.5 and 7.0. No growth occurred at pH 3 and growth declined markedly above pH 7.5. No effect on sporulation was observed, as the number of conidia appeared to be directly related to the size of the colony. The pH of the expressed juice of carrot leaves tested varied from 6.3 to 6.7.

DISSEMINATION IN THE FIELD

Observations in the Santa Maria Valley indicate the probable importance of wind as an agent in the distribution of Cercospora blight inoculum. The prevailing wind is from the west and off the ocean. At harvest, plants along the east side of a field of infected carrots usually are more severely blighted than those on the west. Sufficient inoculum, apparently, is carried by the wind to increase infection on the east side. Also when there are fields of young carrots on the east and west sides of a field of older badly infected carrots, the former in the east field, next to the infected plants, are the more heavily infected.

During harvest, agar plates were exposed for 3 minutes at 10, 100, and 300 feet distant from the leeward side of a field badly affected with Cercospora leaf blight. The fungus was recovered from the air at all three locations.

Other workers (4, 8, 11, 14) have reported that wind is an important agent in disseminating the species of Cercospora attacking celery, peanuts, and beets.

SEED TRANSMISSION AND SEED TREATMENT

The seed umbels of carrot plants growing in an experimental plot at Santa Maria were found affected with Cercospora blight. Seeds saved from these plants had conidia of the fungus adhering to the seed coat. A few of the local growers at Santa Maria save a small quantity of seed for their own use. To determine if the locally-grown seed was contaminated with the fungus, portions of 8 seed samples were washed in 10 cc. of water and the liquid drained off and centrifuged. The precipitate and several cc. of liquid remaining in the bottom, after the supernatant liquid was poured off, were shaken up and sprayed on carrot leaves in the greenhouse at Berkeley. A few Cercospora leaf spots were found 14 days later on plants inoculated with 4 of the 8 samples. No infection was secured with the wash water from the seed harvested in the area where it is commercially saved nor on unsprayed controls. Repeated attempts to obtain infection from the centrifuged wash water from commercial seed were negative.

As very little locally grown seed is used within the Santa Maria Valley seed transmission is probably not an important source of infection. Although Cercospora is generally absent from the commercial seed fields near Sacramento, where a large part of the commercial seed is grown, the finding of Cercospora in one of these fields by Gardner, Kendrick, and Ark in June, 1940,[3] suggests that contaminated or infected seed occasionally may introduce carrot blight into territories previously free of the fungus. Doran and Guba (1) obtained no evidence that *Cercospora carotae* was carried on the seed. Other workers (4, 5, 6, 12, 14) have cited evidence indicating that seed transmission occurs with species of Cercospora affecting celery, beets, dill, soybeans, and peanuts.

Several treatments were applied to carrot seed to determine those that might be used without seed injury and would be effective both in controlling damping-off and freeing the seed of fungous and bacterial contaminants adhering to the surface. Seven replicates of 50 seeds from each of 14 treatments were sown in unsterilized soil in the greenhouse. The plots were arranged in a modified latin square. Stand counts were made 10 days after seeding. Compared with the stand of 33 per cent for the nontreated check the stands from seed treated with the following were significantly better: mercuric chloride (1–1000 for 5 min.), 49 per cent stand; cuprous oxide dust, 47 per cent; malachite green (1–2000 for 5 min.), 45 per cent; zinc oxide dust, 52 per cent; copper sulphate (2 oz. to 1 gal. for 1 hr.), 60 per cent; Spergon (dust), 60 per cent; Spergon (1–1200 for 5 min.), 45 per cent, and ethyl mercury tartrate (1 per cent preparation used as a dust), 55 per cent. Ethyl mercury phosphate (5 per cent preparation used as dust), 20 per cent; significantly decreased germination. Mercuric chloride (1–2000 for 5 min.), 41 per cent; mercuric chloride (1–3000 for 5 min.), 37 per cent; ethyl mercury tartrate (1–24,000), 35 per cent, and ethyl mercury phosphate (1–24,000), 33 per cent, did not significantly affect the percentage germination compared with the nontreated check.

[3] Letter from M. W. Gardner, dated October 17, 1940.

The effectiveness of the above treatments in freeing the seed from disease inoculum and other fungous or bacterial contaminants was determined by dipping carrot seed into a suspension of Cercospora conidia, drying the seed, treating, and then placing 25 seeds from each treatment on potato-dextrose agar plates. Four days later the seed treated with ethyl mercury phosphate and ethyl mercury tartrate, either as a liquid or a dust, mercuric chloride (1–1000) and Spergon (dust) were free from any fungous or bacterial growth. Dilutions of 1–2000 and 1–3000 of mercuric chloride sterilized 92 and 96 per cent of the seed, respectively. The other treatments were ineffective. It is possible that the active fungicidal ingredients in the treatments diffused into the agar and only inhibited the germination and growth of the contaminants adhering to the seed.

PERSISTENCE IN SOIL

In the fall of 1937, Cercospora lesions were found on the first foliage leaves of 3-week-old carrot plants in several fields at Santa Maria, where blight had been serious on previous carrot crops. This suggested that infection came from the soil.

In July, 1938, soil samples were taken from the surface, 4 inches deep, and 6 inches deep in a field where the carrot crop harvested 2 months earlier was severely infected with Cercospora blight. These soil samples were placed in sterilized pots and planted to carrot seed in a greenhouse at Berkeley in which carrots had never been grown before. The pots were covered with a moist chamber, which was not removed until the young carrots were several inches high. No Cercospora infection was found on the plants. The pots were then filled with water and the surface of the soil stirred until the water was muddy. The tops of the plants were bent over and dipped into this muddy water. A moist cheese cloth cover was placed over the plants for 48 hours. At the end of 20 days, in the samples taken at the surface, 3 plants out of 1654 were found with sporulating Cercospora lesions. No infection was found on carrots planted in similar soil sterilized before use.

In contrast with certain other carrot-producing sections, there are carrots in the field at Santa Maria during the entire year. Often a crop is not harvested at maturity because of unfavorable market conditions. For example, carrots ready to harvest in December, 1936, were not harvested until February and March, 1937. Conidia of Cercospora were abundant at harvest on the older dead leaves of these plants and could provide a source of infection for the spring crop planted in nearby fields.

Infected carrot leaves were collected in December, 1936, and placed in wire containers. Some of these were placed on the surface of the soil, some 4 inches deep, some 6. These containers were removed in September, 1937, and typical Cercospora was secured on plants inoculated with water suspensions of the decayed leaves.

The possibility of the fungus establishing itself in the soil in the absence of a carrot crop was investigated. A suspension of Cercospora conidia was

placed in the center of Petri dishes filled with sterile soil. Upon examination later it was found that mycelium had developed and produced conidia. Similar results were obtained with unsterilized soil. Considerably more growth was made on soil high in organic matter than on soil of a sandy texture typical of that on which carrots are raised in the Santa Maria Valley.

Doran and Guba (1) have reported that *Cercospora carotae* hibernates in dead leaves of carrots in or on the soil; other workers (4, 7, 8) found that other parasitic members of this genus overwinter on the dead leaves of their hosts.

RELATION OF TEMPERATURE AND HUMIDITY TO THE SEVERITY OF CERCOSPORA BLIGHT

Cercospora blight proved most serious at Santa Maria during June, July, and the first 2 weeks of August. Very little infection was found from the middle of August until March, when the disease began to appear generally in the valley. The damage caused early in the spring was slight, and it was June before any appreciable amount of infection developed.

During the summer and early fall very little rainfall normally occurs, but periods of very foggy weather are common. Carrot leaves are frequently covered with moisture from 6 p.m. until 8 a.m. There is little or no increase in the severity of Cercospora blight when the rainy season begins in the fall.

The factor or factors responsible for the decreased activity of Cercospora blight during late August and early fall have not definitely been determined. Hygrothermograph records (kept from August, 1936, to July, 1937) at Santa Maria show that during the period from April to July, when Cercospora was most active, the average minimum temperature fluctuated from 40° to 50° F. and the average maximum between 70° to 77° F. From July to November, the average minimum temperature ranged from 48° to 57° F. and the average maximum from 70° to 81° F. Laboratory tests showed that the fungus grew most rapidly on agar between 66° and 79° F

THE EFFECT OF THE MINERAL NUTRITION OF THE CARROT PLANT UPON THE DEVELOPMENT OF CERCOSPORA BLIGHT

The effect of the mineral nutrition of the carrot plant upon the development of Cercospora blight was determined by counting the number and classifying the length of the leaf spots and by estimating the abundance of conidia produced on the leaf spots on the plants grown in solutions complete in all nutrients, or deficient in nitrogen, phosphorus, potassium, or calcium.

Three carrot plants of the variety Morses Bunching were grown in 6-inch, glazed pots, 5 pots for each of the 5 treatments. The pots were arranged in a latin square. A complete nutrient solution, made up according to Hoagland and Arnon's formula 1 (3) was supplied to the plants by a continuous drip mechanism, similar to that described by Pryor (10). When the plants had from 4 to 6 leaves, the pots were flushed with several liters of distilled water, and solutions deficient in nitrogen, potassium, phos-

phorus, or calcium, respectively, made up according to Hoagland and Arnon (3), were supplied to the plants. Extra carrot plants in each treatment were used to determine when deficiencies of nitrogen, phosphorus, and potassium occurred, by means of the plant tissue tests described by Thornton, Conner, and Fraser (13).

After deficiency symptoms were evident, the plants were sprayed with a suspension of Cercospora conidia from carrot leaves and placed in a moist chamber at 75° to 85° F. for 72 hours. Fifteen days after inoculation, the number of leaf spots per plant, length of leaf spots, and number and fresh weight of infected leaves were recorded. The approximate leaf area was determined by dividing the leaf weight by the weight per cm.^{2} of leaf blade. The latter figure was determined by tracing around carrot leaves on paper, dividing the weight of the paper by the weight of one square centimeter of

TABLE 1.—*Influence of the mineral nutrition of the carrot on susceptibility to Cercospora carotae*

Treatment	No.[a] of leaves	Area of leaf	No. of leaf spots per cm.^{2}	Leaf spot length	Conidia production[b]	Weight of the fungus mat on leaf extracts[c]
		Cm.^{2}		*Mm.*	*Class*	*Mg.*
Complete	9.6	93.8	0.31	6.5	5	700
−K	7.1	33.3	0.28	4.7	4	750
−P	7.0	20.7	0.26	4.9	3	470
−Ca	5.9	44.6	0.19	2.7	2	270
−N	7.6	26.3	0.19	2.3	2	830
Significant difference 5% level	2.5	5.1	0.06	0.87	...	210

[a] Composite figures for two experiments. An average of 10 plots, arranged in a latin square.

[b] Abundance of conidia produced on leaf spots on plants placed in humidity chamber for 36 hours. 0 = no conidia, 5 = many conidia.

[c] Growth of *Cercospora carotae* made in 60 days in a sterile leaf extract of plants grown in deficient solutions. Average of 5 flasks.

paper, and then dividing the weight of the leaves by this figure. The average number of leaves, average area per leaf, average number of leaf spots per cm.^{2}, average length of the leaf spots and abundance of conidia on the leaf spots are summarized in table 1. The data from the two experiments have been analyzed together by the analysis of variance method and only the composite results are presented.

Potassium

Six days after being given the potassium-deficient solution, the potassium concentration of the leaf sap was low. However, it was approximately 10 days before potassium deficiency symptoms were evident. They consisted of a purplish tinge on the underside of the leaves and the development of new leaves that were smaller than those of plants on the complete solution. A deficiency of potassium did not significantly affect the number of leaf

spots but did significantly limit the length of the spots. The abundance of the conidia on the leaf spots as observed through the binocular microscope was less.

Phosphorus

The first signs of phosphorus deficiency, which became evident after approximately eight days, were a stunting of the new growth and a purpling of the older leaves, particularly along the veins. The new leaves developed a darker green color. The number of leaf spots was not significantly affected, but the length of the leaf spot was significantly limited. The conidia were less abundant on the phosphorus-deficient plants than on either the complete-nutrient or potassium-deficient plants.

Calcium

The characteristic symptoms of calcium deficiency were, in the first experiment, a stunting and chlorosis of the new leaves within 12 days after transference of the plants to the calcium-deficient solution, followed by a necrotic spotting of the leaves and tip necrosis five days later. At this point one millimol of calcium chloride was added to the solution to prevent further necrosis. In the second experiment the calcium chloride was added as soon as the stunting of the leaves was noticed to prevent development of necrotic symptoms. The number of leaf spots was significantly reduced. Approximately two-thirds as many were found as on the plants on the complete solution. The leaf spots were less than one-half as long, and the abundance of the conidia was approximately one-half that of the plants on complete nutrient.

Nitrogen

Seven days after the plants were placed in the nitrogen-deficient solution the plant tissue test first indicated that the leaves were low in nitrate nitrogen. The new leaves were reduced in size, and both the new and old leaves were yellowish-green in color. The nitrogen-deficient plants were very similar to the calcium-deficient plants in behavior to infection. The number of leaf spots was only two-thirds that of the complete-nutrient plants. The leaf spots were only about one-half as long. The conidia were approximately one-half as abundant as on the complete-nutrient plants.

GROWTH OF CERCOSPORA CAROTAE ON LEAF-JUICE EXTRACTS OF PLANTS GROWN IN VARIOUS MINERAL SOLUTIONS

The growth of the fungus on sterile juice extracts of plants grown under the different nutrient conditions was determined and compared with the development of the disease on such plants.

Carrot plants were transplanted into five 14-inch glazed pots filled with white silica sand and grown in a complete-nutrient solution until they had approximately ten leaves. The sand then was flushed with distilled water and the following solutions supplied, one to each pot: complete-nutrient,

potassium-deficient, phosphorus-deficient, calcium-deficient, and nitrogen-deficient.

When deficiency symptoms had developed, the leaves were ground, the juice extracted with a fruit press, and then diluted by one-third with distilled water. This mixture was sterilized by passage through a Seitz filter. Fifty cc. of the sterile extract was transferred aseptically to each of five 125-cc. Erlenmyer flasks. These were then inoculated with a suspension of conidia and stored at room temperatures for 2 months. *Cercospora carotae* grew very slowly in these extracts, and formed only a small mat in this period. The contents of each flask was filtered and the weight of the oven-dried fungus mat obtained.

There was significantly less growth made on the extracts from the phosphorus and calcium-deficient plants as compared with the complete-nutrient, potassium-deficient, and nitrogen-deficient plants. No correlation was found between the weight of the fungus mat on the extracts from the plant and the number of leaf spots, length of leaf spot, or number of conidia produced (Table 1).

SUMMARY

Cercospora carotae attacks the leaf blades and petioles of the carrot (*Daucus carota*), and *Daucus maritimus, D. pulcherrimus, D. pusillus, D. hispanicus* and *D. gingidium.* No marked resistance to Cercospora blight was found among 112 carrot varieties, selections, and *Daucus* species tested in the field.

The germ tubes enter the host through the stomata. The advancing hyphae are intercellular at first, but intracellular invasion soon occurs. Sporulation may occur soon after the fungus establishes itself in the substomatal cavity, but more often after the mycelium has invaded more deeply into the host. The fungus invades all of the epidermal and parenchymatous tissue between the two surfaces of the leaf blades, but in the petiole seldom penetrates very deeply until the leaf begins to die.

Most rapid growth of the fungus in culture occurred between 19° and 28° C. Maximum germination of conidia occurred between 16° and 28° C., and abundant conidia production between 13° and 28° C. Maximum growth in culture was made between pH 5.5 and 7.0.

The fungus may persist in the soil from one crop to another and may be spread to other fields by wind.

Infected seed umbels have been observed and viable conidia are found on the seed from such plants. Carrot seed, artificially contaminated with conidia of the fungus, treated with ethyl mercury phosphate and ethyl mercury tartrate either as a liquid (1–24,000) or a dust, mercuric chloride (1–1000) and Spergon remained free of fungous growth when placed on sterile potato-dextrose agar. The effect of several seed treatments on percentage stand has been determined in the greenhouse.

Number of leaf spots per cm.^{2} did not differ significantly on carrots complete in nitrogen, phosphorus, and potassium, deficient in potassium, or

deficient in phosphorus. The calcium-deficient and the nitrogen-deficient plants had significantly fewer leaf spots per cm.^{2} when compared with the complete-nutrient plants. The leaf spots attained their greatest length on the complete-nutrient plants, were intermediate in length on the potassium-deficient and the phosphorus-deficient plants, and were shortest on the calcium-deficient and the nitrogen-deficient plants. Conidia were most abundant on the lesions on the complete-nutrient plants, less abundant on the potassium-deficient plants, and least abundant on the phosphorus-deficient, the calcium-deficient, and the nitrogen-deficient plants.

When the fungus was grown on the sterile leaf-juice extracts obtained from plants deficient in potassium, phosphorus, calcium, or nitrogen and plants complete in these elements, more growth was obtained on the leaf extracts from the complete, the potassium-deficient, and the nitrogen-deficient plants than on extracts of the calcium-deficient and the phosphorus-deficient plants.

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THE PRESENCE OF A TOXIN IN TOMATO WILT[1]

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INTRODUCTION

Although the fungi that cause many vascular wilts are well known, the mechanism by which wilting occurs remains a moot problem. Many theories have been formulated to account for loss of turgidity by the plants; the earliest of these hypothesized the plugging of vessels. Massee (14), working on the sleepy disease of tomatoes, and, shortly afterwards, Smith (17), investigating the wilts of cotton, watermelon, and cowpea, observed that many of the tracheae in the hosts were filled with hyphae. From this evidence they postulated the theory that water was unable to pass through the xylem in sufficient quantities to keep the leaves turgid. Therefore, the plants died of drought. Brandes (4), and Ahmet (1), however, point out that mycelium usually is not present in sufficient quantities to hinder the passage of water in the tracheae. Clayton (5) tested the plugging hypothesis by girdling portions of tomato stems, but was unable to obtain a unilateral wilt, such as has been observed often in the field. Because of the resemblance borne by tomato wilt to the symptoms obtained when poisons are injected into plants, Clayton believed that toxins produced by the pathogen caused the wilting. Evidence was slowly accumulated that many of the wilt organisms produce poisons in synthetic media (2, 5, 6). When stems of healthy plants were placed in the filtrates of nutrient solutions in which the pathogen had been grown, they wilted; and the vascular tissues were discolored. From such data the theory that a toxin produced by the fungus causes vascular wilts has gained credence. In this paper the term toxin is not used in its strict medical meaning, but will indicate a poisonous substance produced in the plant due to the presence of the fungus. Good reviews of literature on the subject of fungus toxins are given by White (20), Grossman (9), and Fisher (8). Many investigations have been made concerning the nature of the toxin found in the synthetic media, but the active principle has not yet been identified (7, 13, 16). Still another explanation of the cause of wilting has been advanced by Tochinai (18). In a study of the physiology of *Fusarium lini* he discovered that a large amount of carbon dioxide was produced by the parasite. This, he claimed, could form gas pockets in the vessels, which might stop the movement of water through the stem, thereby causing the wilting of flax.

Evidence for the existence of fungal toxins has been obtained by growing the fungi in synthetic media. Such evidence is not entirely conclusive. It is well known that materials produced by fungi will vary with the substrate

[1] Paper No. 2003, Scientific Journal Series, Minnesota Experiment Station. Assistance in the preparation of figures 2, 3, 5 was furnished by the personnel of the Works Projects Administration, Official Project No. 165–71–1–124, Sponsored by the University of Minnesota (1942).

on which they are grown (3). Rosen (15), for example, found that *Fusarium vasinfectum* produces a toxic substance when grown in Richards' solution, but not when grown in nutrient broth or Uchansky's solution. Since the environment and nutrients in the tracheae of a plant are very different from those of a synthetic nutrient solution, there is a possibility that no toxic materials or that different toxins are produced by the fungus in the host plant. Hursh (12) sums up the present status of the problem very aptly:

"It was postulated that the pathologic condition in plants infected with certain wilt disease might be brought about by some fungus products deleterious to the host tissue that had been transported considerable distances through the stem from the point of attack. Should the presence of such

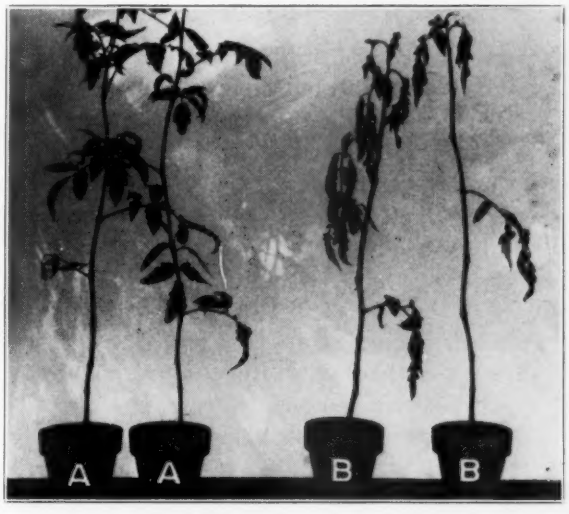

FIG. 1. Tomato plants used for the extraction of tracheal fluid. A. Noninoculated turgid plants. B. Inoculated wilted plants.

substances actually be demonstrated it would be an important contribution to an understanding of the physiology of fungus parasitism." With this in mind, the object of the present research was to ascertain the presence or absence of toxins as a factor in the fusarial wilt of tomatoes.

MATERIALS AND PROCEDURE OF THE PRESENT WORK

The Bonny Best variety of tomato was used as the host and a virulent isolate of *Fusarium bulbigenum* var. *lycopersici*[2] as the pathogen. Tomato seeds were planted in flats containing a steam-sterilized mixture of 2 parts silt loam and 1 part sand. After the first true leaves appeared, the seedlings were transplanted to 4-inch pots and grown until approximately 45 cm. tall. The plants were then inoculated with a culture of the *Fusarium* which had been grown in Richards' solution for two weeks. Wellman's (19) method

[2] The isolate of *Fusarium bulbigenum* var. *lycopersici* was obtained through the courtesy of Dr. F. L. Wellman, U. S. Department of Agriculture.

of inoculation was used, and the plants were immediately repotted. They were covered with manila paper for 48 hours before exposure to direct sunlight. Unless this precaution was taken the tomatoes received a severe shock, resulting in injury that might have been confused with the disease. The temperature in the greenhouse varied from 75 to 87° C., and moisture in the soil was kept just under the saturation point. Check plants were treated similarly except that their roots were placed in sterile water instead of in the inoculum. Within 21 days after inoculation most of the inoculated plants had wilted, whereas the checks remained turgid (Fig. 1). The vascular systems of all the diseased plants were discolored; those of the healthy plants were not.

THE EXTRACTION OF PLANT-CELL SAPS AND WILTING OF TOMATO SEEDLINGS IN THE EXTRACTS

The tomato plants were placed in a freezing chamber for 24 hours. The leaves were then stripped off and the stems cut into small sections. A hydraulic press was used to express the sap under pressure ranging from 250 lb. to 15,000 lb. per sq. in. The sap was next centrifuged at 2500 r.p.m. for 30 minutes and the supernatant fluid freed of the pathogen by passage through a Berkefeld filter. The filtered sap was transferred to small tubes 5 mm. in diameter into which the shoots of tomato seedlings 10 cm. tall were placed and observed for wilting.

No consistent differences in time required for wilting could be observed between the seedlings in the sap of diseased plants and those in the sap of healthy plants. Both saps caused wilting within 4 hours. An absolute check of sterile water also was run. No wilting, however, was observed in the checks even after 24 hours. To determine whether the wilting in the sap might be due to an effect of osmotic pressure, the sap was diluted as much as 1 to 8 with distilled water. Even in such low concentrations, however, the plants wilted within 4 hours. This indicated that some factor other than a high osmotic value was responsible for the toxic condition of the saps of both normal and diseased plants when they were thus extracted. Sap obtained at a pressure of 250 lb. per sq. in. proved less toxic than that obtained at a pressure of 15,000 lb.

It was apparent that if any toxin with a capacity for wilting tomato seedlings had been secreted by the parasite in the diseased plant, its action was masked by other injurious substances formed or secured during the extraction of plant saps. Such poisons might be formed by the release of some cellular contents and their chemical change upon death or injury to the plants. In his investigations on wilt of potatoes, Haskell (11) has evidence to support this possibility. He discovered that when a portion of the stem was killed with steam some poisonous materials were formed that discolored the vascular system, as occurs in the fusarial wilts. To preclude the effect of such poisonous agents in the sap, a method was devised for using tracheal fluids rather than the expressed sap.

CENTRIFUGING TRACHEAL FLUIDS

Since the pathogen is a vascular parasite, it seems reasonable to assume that any toxins present should first appear in the vascular fluids. An assay of these fluids should reveal the presence or absence of the toxin involved in tomato wilt. Centrifuging the vascular fluids from the stems of the plants was decided on as offering the most feasible means of obtaining these liquids.

Apparatus

Centrifuge tubes similar to those described by Hamm *et al.* (10) for spectrographic analysis were used. These had been previously modified so

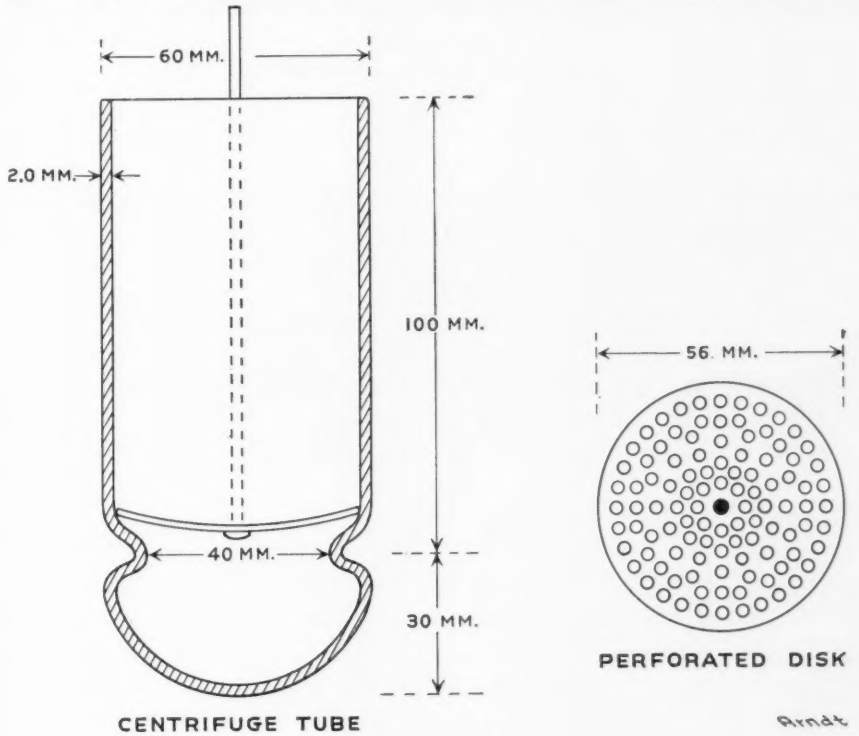

FIG. 2. Centrifuge tube.

that the stems were contained in a glass inner tube with a small hole at the closed end through which the liquid escaped into the collecting chamber. Because of the extremely small amounts of fluid obtained by centrifuging tomato stems, a different type and much larger tube was designed[3]. The vessel was constructed of a section of heavy walled pyrex tube 130 mm. long and 60 mm. in diameter. One end of the tube was sealed to form a rounded bottom, and a collar was blown into the inside of the vessel, 30 mm. from the bottom. This shelf supported a removable stainless-steel plate 55 mm. in diameter and into which 100 2-mm. holes had been drilled. A steel rod was riveted to the center of the plate to facilitate removing the stems from the

[3] The aid of Dr. Richard Nelson in designing this tube is greatly appreciated.

centrifuge tube. The diaphragm was covered with glass wool to filter off any solid particles dislodged from the stems during centrifuging (Fig. 2).

Since chemical changes might occur during the extraction, the entire experiment was run under an atmosphere of nitrogen. The joints of the centrifuge were sealed with glazier's putty and the cover sealed with petrolatum. A water trap was placed between the nitrogen tank and the centrifuge to aid in regulating the flow of gas. The outlet of the trap was provided with a 3-way stopcock; one of the arms led to the centrifuge chamber and the other to a pipette used for flushing the centrifuge tubes free of air before each run. This same outlet was used when filtering the tracheal fluids anaerobically. The entire apparatus is illustrated in figure 3. An

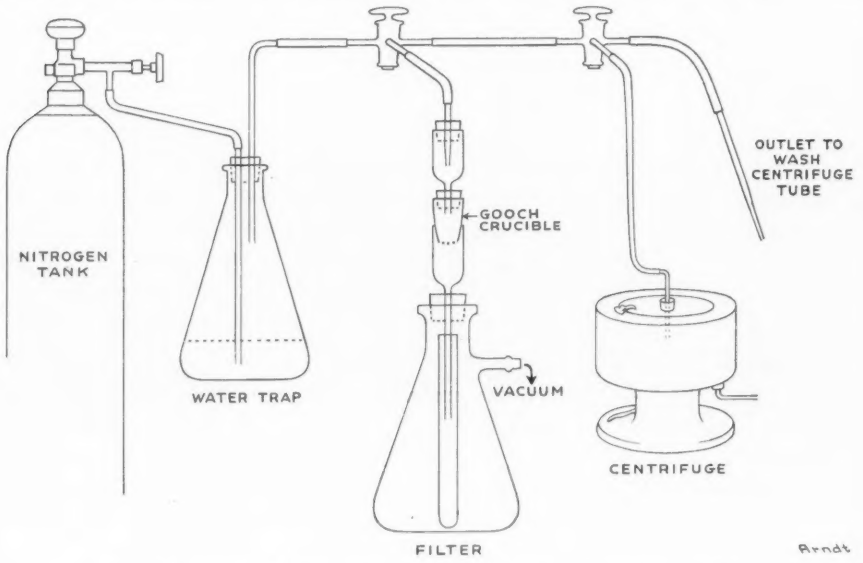

FIG. 3. Apparatus for centrifuging and filtering tracheal fluids.

analysis of the gaseous contents of the centrifuge after some sample runs, showed the presence of only 2.01 per cent of 2.12 per cent oxygen, with an average 2.03 per cent.

Procedure

The plants were grown and inoculated as described in the previous experiment. After they had wilted, the leaves were removed and the stems cut into pieces 8 cm. long. These were packed in the tubes, which were then flushed with nitrogen and centrifuged. The centrifuge was washed with nitrogen for 40 minutes prior to addition of the samples and the gas continued to flow into the machine during the entire extraction. After centrifuging for 20 minutes, the liquid was removed from the collecting tubes and stored under nitrogen at $-18°$ C. for 12 to 24 hours. The tracheal fluids later were filtered anaerobically.

Small vials made of 4-mm. glass tubing, 50 mm. tall, having a capacity

of 1.5 ml., were used to hold the tracheal fluids and the seedlings during the tests. Large corks were found to be excellent bases for them. One ml. of the filtrate was placed in each test vial. Seedlings 3 weeks old and 6 to 8 cm. tall, whose stems had been cut under water, were used to test for the presence of a toxin, one seedling being placed in each vial. These assays were run in the laboratory under an illumination of 405 foot candles obtained from 2 100-w. Mazda lamps at a height of 60 cm. from the table. The temperature varied between 26 and 28° C. The time required for wilting of seedlings in the vials was observed. Tracheal fluids from the uninfected tomatoes were used as checks. To determine whether the tracheal fluid of normal plants affected the seedlings, absolute checks were run with distilled water.

Precautions Taken in Selecting Plant Materials

The plants were examined for vascular discoloration before using them in this work. All 152 of the inoculated plants had brown vascular bundles

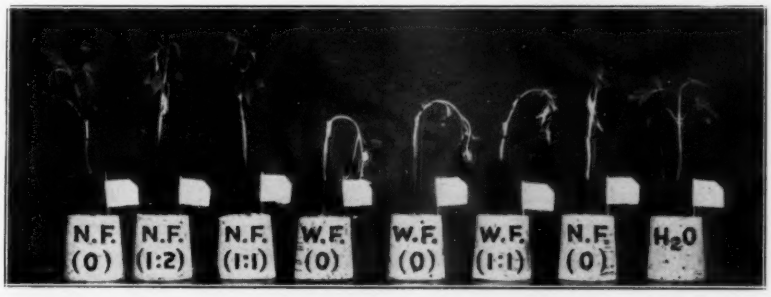

FIG. 4. Assay for toxin. Vials labeled N.F. contain tracheal fluid from noninoculated turgid plants. Vials labeled W.F. contain fluids from plants which were inoculated with *Fusarium bulbigenum* var. *lycopersici* and which had wilted.

at time of wilting. This discoloration was absent in the 103 noninoculated plants. Microscopic examination of free-hand sections from stem and root tissues fixed in Formalin, alcohol, acetic acid solution, showed that the tracheae of the inoculated plants contained mycelium; whereas, no mycelium was found in the noninoculated ones. Isolations on potato-dextrose agar were made from the same plants and a *Fusarium* was obtained from all the material that had been inoculated. No *Fusarium* was isolated from tissues of healthy plants. Mycelium from the isolations was reinoculated into healthy plants and caused a typical wilt.

THE EFFECTS OF TRACHEAL FLUIDS ON TOMATO SEEDLINGS

A total of 42 seedlings, divided into 3-series, was used to determine the toxicity of the vascular fluids. Sixteen seedlings were placed in the extract of the diseased plants, 14 in the extracts of normal plants, and 12 in distilled water. In each series the seedlings in the liquid obtained from the diseased plants wilted long before those in the fluid from the healthy plants, the time required varying from series to series, from 1 hour to 7 hours (Fig. 4); but

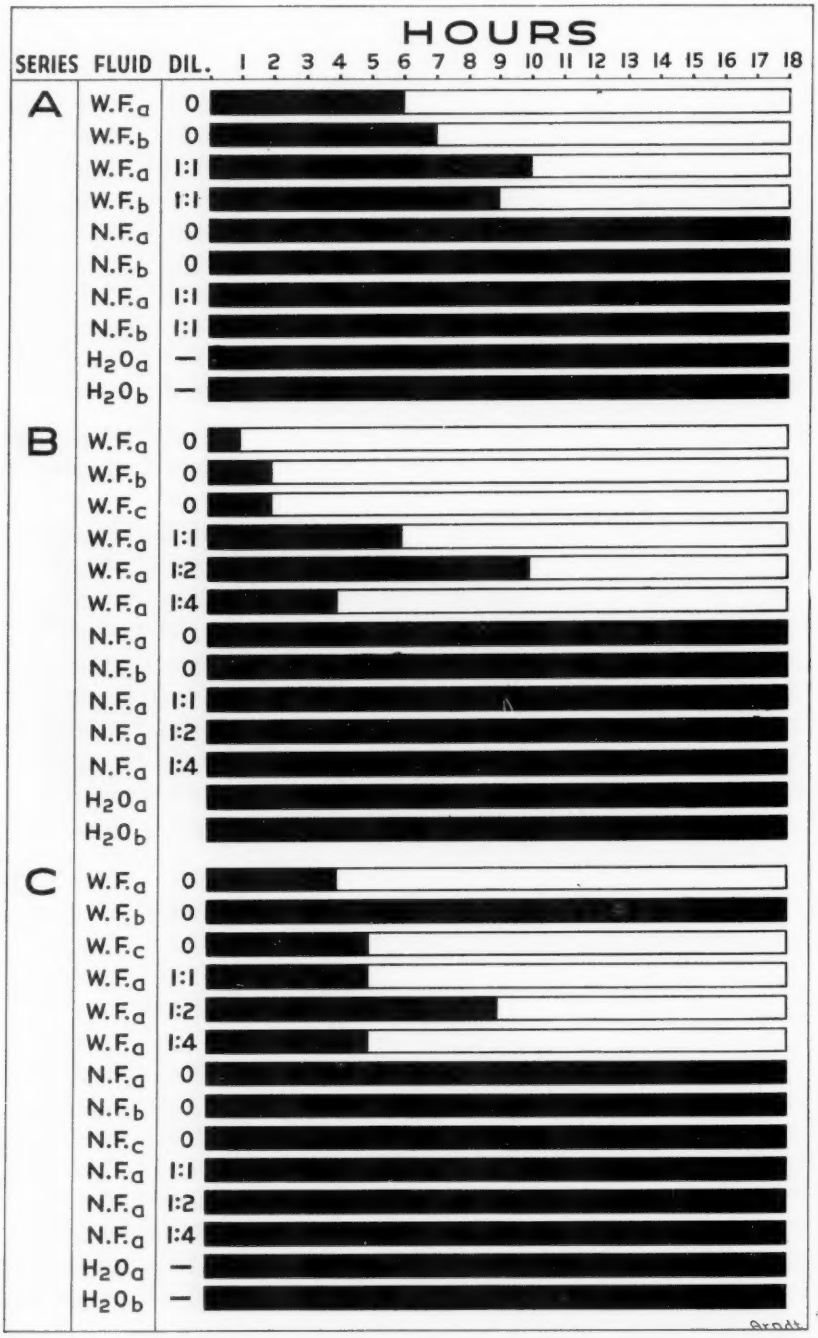

FIG. 5. Chart showing time required for tomato seedlings to wilt. Black bar denotes turgidity; white bar indicates a wilted condition. Fluids N.F. were obtained from non-inoculated, turgid plants. Fluids W.F. were obtained from inoculated wilted plants.

in each series, the replicates lost their turgidity within 1 hour of each other. Only 1 seedling in 1 series, W.F.$_b$ (0 dil.) in series C, failed to wilt; it was only slightly flaccid even after 19 hours. All the seedlings in the fluids of normal plants and those in distilled water remained turgid during the entire course of the analysis (Fig. 5). Dilution of the fluids from diseased plants affects wilting. Although seedlings wilted much sooner in a zero dilution of the extract than in greater dilutions, there was no significant correlation between the rate of dilution and the rapidity of wilting. The results indicate that there is some toxic material in the tracheal fluids of tomato plants infected with *Fusarium bulbigenum* var. *lycopersici*.

TRACHEAL FLUIDS FROM PHYSIOLOGICALLY WILTED PLANTS

Because of the chance that the toxic character of the tracheal fluids was due to a generalized physiologic reaction of the host to the process of wilting, and not specifically to the presence of the parasite, another series of experiments was planned. In this series the vascular fluids of normal plants were compared with those of tomatoes which had been deprived of water so that they were in an advanced stage of wilting. Eight seedlings were placed in each of the different fluids. All the seedlings in the extract from the normal, turgid plants and all in the extract from tomatoes which had wilted because of insufficient water, remained turgid even after 24 hours. Thus, a physiological wilting in itself does not result in the presence, in tracheal fluids, of materials toxic to young tomato seedlings. We may justly conclude that the toxin present in tracheal fluids from plants wilted by *Fusarium bulbigenum* var. *lycopersici* must be due to the activity of the fungus.

DISCUSSION

From the results obtained in this investigation, it is evident that a toxin is present in the tracheal fluids of tomatoes showing fusarium wilt. The active constituent, moreover, is associated definitely with the presence of the fungus, *Fusarium bulbigenum* var. *lycopersici*, for, when wilting was induced in absence of the pathogen no toxin could be found in the fluids. This evidence is in line with past researches showing that the fungus produces poisonous substances in artificial media. However, the identity of the toxins in the tracheal fluid and those in the synthetic media has not been ascertained. Rosen (15) has criticized the evidence obtained by growing the pathogen in nutrient solutions. He found a toxin when *Fusarium vasinfectum* was grown in some synthetic media but none when grown in others. He, therefore, raised the following questions as to criteria for valid evidence: "It opens up the whole question as to the significance to be attached to finding of toxic properties with any medium which does not closely approach the chemical and physical makeup of the natural host."

The techniques used in the present study for demonstrating the role of toxins in tomato wilt ought to fulfill, to some extent, these criteria. Since the fungus inhabits the lumen of the xylem, the xylem fluid is the natural medium for the growth of the parasite and the production of toxin. In

addition, the method of extraction precluded, so far as possible, the opportunity for chemical changes of the fluids.

Still, there is a chance that the toxic material is not a direct product of the fungus metabolism. This component of the vascular fluid might result from host cells killed or injured and disorganized by the penetration and progress of the fungus. Haskell's (11) evidence for the production of poisonous substance by steam-injured stems indicates this possibility must not be overlooked. However, separation of the direct and indirect effects of the fungus would be exceedingly difficult to demonstrate.

The centrifuging technique should prove valuable in the study of vascular parasites, such as are involved in Dutch Elm disease, Verticillium wilt of maple, potato wilt, and many others in which toxins are suspected. By this means the tracheal fluids can be obtained with a minimum of mixture with cell sap and with relatively little chemical change. Among its advantages over the sap-displacement procedure are the short time required for the extraction, the fact that there need be no dilution with water, and that the entire process can be run under anaerobic conditions. In addition, the procedure can be adapted for herbaceous as well as woody plants. The only requirements are that the stem be sufficiently sturdy and that there be a sufficient number of vessels with a bore large enough so that the forces of capillarity can be overcome by centrifuging. Therefore, it could not be used successfully on such crops as wheat and flax.

SUMMARY

A method has been devised for collecting anaerobically the tracheal fluids of plants.

Tomato plants wilted by *Fusarium bulbigenum* var. *lycopersici* contain a toxin in their vascular fluids.

No toxins were found in tomato plants wilted by lack of sufficient soil water.

When the cell sap of either healthy tomato plants or those with Fusarium wilt is expressed from the stems under aerobic conditions, a poisonous substance is present that causes tomato seedlings to wilt when placed in the sap.

The writer wishes to express his thanks to Dr. E. C. Stakman and Dr. Helen Hart for their many helpful suggestions; to Dr. Ian W. Tervet for his interest and encouragement during the course of the research; and to Betty Gottlieb for her aid in carrying out this investigation.

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STAMEN BLIGHT OF BLACKBERRIES[1]

S. M. Zeller and Alvin J. Braun

(Accepted for publication May 28, 1942)

Stamen blight of blackberries caused by *Hapalosphaeria deformans* Sydow was first observed in Oregon in 1937 on Young dewberry grown at Kellogg, Douglas County (5). The wild Trailing blackberry (*Rubus macropetalus* Doug.) growing along nearby fences also was infected. The following year infected Young and Boysen dewberries were discovered by F. D. Bailey at North Albany. A year later scattered infections were observed on several commercial plantings of Evergreen blackberry (*R. laciniatus* Willd.), in Marion County. The disease is widely distributed throughout the Willamette Valley on Young and Boysen dewberries, and the Evergreen blackberry.

Stamen blight of Loganberries was reported in British Columbia by Dearness and Foster in 1933 (3). In a recent communication from Glenn A. Huber of the Western Washington Experiment Station (Puyallup) record is given of *Hapalosphaeria deformans* on the Young (Olympic) variety of blackberry in the Puget Sound area. It is evident, therefore, that this disease is widely distributed throughout the Pacific Northwest, especially west of the Cascade Mountains.

In Europe the first published record of the stamen-blight organism was by H. and P. Sydow (1) under the new name *Paepalopsis deformans* (1907). The type locality was Thuringia, Germany, on *Rubus caesius* L. (syn. *R. dumetorum* Weihe). There is also a report (4) of this disease on *R. fruticosis* L. in England in 1904. Diedicke and Sydow (2) first illustrated the fungus within anthers.

SEVERITY OF THE DISEASE

The most abundant infection we have observed involved about 70 per cent of Young dewberry blossoms in a 6-acre commercial planting in 1937. The long life of such plantings and their concentration in culture ordinarily should favor cumulative epidemics in succeeding seasons. Seasonal epidemics of this pathogen, however, are greatly influenced by the fluctuation of aerial environments. Thus in 1938 the above-mentioned planting showed but 15 per cent infection and up to about 30 per cent one season since. Consequently, when conditions are favorable stamen blight has potentialities for serious berry-crop losses.

DESCRIPTION OF THE DISEASE

In the spring, when the flowers of the host open, the fungus is most readily detected. When the petals first unfold the diseased anthers already have a moldy appearance due to white masses of spore coils (Fig. 1). Soon after

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FIG. 1. Young dewberry flowers in two stages of blooming. The two above infected with *Hapalosphaeria deformans*. Note the snowy-white coils of spores exuding from pycnidia in the anthers. These cirri are especially prominent immediately after the flowers open. Note also that the infected anthers do not dehisce, as do the healthy (lower right).

FIG. 2. A. Cross section through a healthy Young dewberry anther. B. Cross section through a Young dewberry anther infected with *Hapalosphaeria deformans*, showing the mycelium in the tissue and between the pollen grains, as well as the stromatic pseudoparenchyma lining the pollen chamber and upon which the spore-filled pycnidia are seated. (Both drawings by Mrs. D. P. Rogers; both ×120.)

the healthy flowers open, their anthers dehisce, exposing cream-colored pollen. In contrast, diseased anthers never dehisce but are covered with white spore horns of the fungus.

The diseased blossoms, completely emasculated by the fungus, usually do not develop normal fruits, since blackberry flowers generally are self-pollinated. Some of these bee-pollinated flowers may produce deformed berries. These are usually unsuitable for canning (Fig. 3).

FIG. 3. Poorly developed berries from blossoms of Young dewberry infected with *Hapalosphaeria deformans.*

Diedicke and Sydow (2) report that *Hapalosphaeria deformans* causes a type of witches' broom in the fruiting laterals of *Rubus caesius.* This proved true also of infected laterals of *R. macropetalus* in Oregon. There is no evidence, however, of such anomalous growth of infected axillary laterals of fruiting canes of Boysen dewberry, Evergreen blackberry or Young dewberry.

The fact that the flowers on a fruiting lateral usually are all infected indicates infection of the unfolded axillary bud. An individual fruiting cane, however, may bear both healthy and diseased laterals.

THE CAUSAL FUNGUS

In 1907, H. and P. Sydow (1) first described the hyphomycetous form of the causal fungus as *Paepalopsis deformans*, on *Rubus caesius* L. The following year Diedicke (2) discovered pycnidia in the anther walls, and Sydow (2) created the new monotypic form genus, *Hapalosphaeria*, in the Sphaeropsidales to accommodate *P. deformans*.

The mycelium in the anther tissues is hyaline, septate, 2–4 μ diam., irregularly branched, and confined to the intercellular spaces. Strands of mycelium form a loose network among the pollen grains within the locules of the anthers. The fungus forms a pseudoparenchyma about 2–5 cells thick completely lining the walls of the pollen sacs (Fig. 2, B). This not only prevents the dehiscence of the anthers and escape of the pollen but also the complete infection of the anther walls starves the developing pollen. Pycnidia with brownish walls are formed in the anthers seated on the pseudoparenchymatous layer and opening to the outside of the anthers. Upwards of 50 pycnidia may be found in one anther.

The subglobose or ovoid pycnidia, 50–110 μ in diameter, are filled with subglobose, hyaline pycnidiospores which are about 2.5–4.9 μ in diameter. These spores are discharged in white coils, which give the anthers the white, moldy appearance (Fig. 1) mentioned above.

The fungus may be readily cultured on potato-dextrose agar. Anthers dissected aseptically from closed buds parasitized by the fungus have usually yielded pure culture of *Hapalosphaeria deformans* when planted on the above medium. Pycnospores from such flowers when planted in agar Petri plates also give pure cultures.

Pure cultures grow slowly on agar, and thus the colonies form firm, compact, dark-gray mats. The agar directly beneath these mats and around their edges becomes discolored with shades from yellow to deep-Burgundy, each isolate imparting to the agar its own particular hue throughout a series of subsequent transfers. At times a whiter strain may be isolated but, so far as known, these light-colored strains may not differ in other respects from the usual dark-gray strain.

No spores or sclerotia have been formed in the pure cultures of the fungus, even during periods as long as 1 year.

Infected Young dewberry anthers were overwintered in cloth bags on and above the ground in an attempt to discover a perfect stage of *Hapalosphaeria deformans*. No such stage was found. After 3 years of unsuccessful attempts it is concluded that if there is a spore form other than the pycnidial stage it is formed very rarely and under unusual conditions. At any rate, it can be assumed that the perfect stage is not essential to the annual epidemiology of the disease, especially as indicated by field experiments

reported later in this paper. Anthers that were not badly contaminated with mold after exposure to field conditions during the winter, readily yielded cultures of *H. deformans* when plates were poured from agar suspensions made by crushing the anthers. This indicates a rather long life of the thin-walled pycnidiospores, at least when protected within the pycnidia.

EXPERIMENTS ON FIELD INFECTION

After unsuccessful attempts to obtain a perfect stage of the causal organism during two winters, it seemed probable that the pycnidiospores produced in the anthers at blossoming time must be capable of infecting the axillary buds of the current-season canes. Two experiments were set up at Kellogg, Oregon. In one experiment axillary buds of Young dewberry were dusted with spores from infected blossoms at the peak of the 1940 flowering season, when the new cane growth was about 3 to 4 feet long.

Approximately 75 per cent of the blossoms had opened by May 8. At this time 5 buds per cane on each of 40 canes were inoculated by lightly tapping an infected blossom against an axillary bud just becoming evident in the axil of the leaf. A good spore load for each bud was obtained by using 1 infected blossom for each set of 2 or 3 buds. Each of the 40 canes was tagged for identification. On May 9, 1941, a count of the number of infected laterals growing from the dusted axillary buds was made. The results of this experiment are given in table 1.

TABLE 1.—*Data taken May 9, 1941, on flowering laterals of Young dewberry originating from axillary buds dusted with pycnidiospores of Hapalosphaeria deformans the preceding year (May 8, 1940)*

Plant structures dusted	Total counted	Infected	Percentage of infection
(1) Flowering laterals from axillary buds dusted with spores in 1940	127	53	41.7
(2) Flowering laterals from axillary buds not dusted with spores in 1940. [Same plants as in (1)]	1000	55	5.5
(3) Natural infection of flowering laterals in plants near those dusted with spores	1000	55	5.5

In a second experiment all of the canes, young as well as fruiting, were removed from a row of 24 Young dewberry plants in a portion of the field showing 21 per cent infection. The canes were cut out on August 2, 1940. Since the new canes, produced late in the season, attained lengths of not more than 2 to 6 feet, they supported relatively few axillary buds. Five hundred flowering laterals from these canes, observed on May 9, 1941, revealed *no* infection, whereas plants normally treated on either side showed from 6 to 8 per cent infection.

The data presented in table 1, as well as the results of the pruning ex-

periment, indicate rather conclusively that axillary buds of current-season canes are infected directly by pycnidiospores or perchance by sporidia produced from them. It may also be observed from these experiments that the source of inoculum of this disease probably is limited to the pycnidiospore or its derivatives. The period of inoculum production is essentially that of and just following the blossoming period of the host plant. Since, as stated above, pycnidiospores may withstand the drouth of summer and the rigors of winter, it is not yet known just when bud infection by *Hapalosphaeria deformans* actually takes place between May and the following March. It seems probable that the pycnidiospores lie dormant within the leafy scales of the winter buds and do not germinate until late in February or early March.

Since the blackberry does not possess a tightly closed scaly bud, infection undoubtedly occurs through the more or less open apex. The first evidence of bud infection, as observed through a study of histological sections, was seen in March. A slight, scattered network of mycelium between the flower buds within the axillary winter bud was then discovered. Then, within the flower buds, a very sparse growth of mycelium may be found among the flower parts. Histological sections, however, have revealed no floral parts infected, except the anthers; nevertheless hyphae may be found growing over and between the pistils in later stages. Since there is no infection of the stamen filaments, incipient infection of the host tissues must be accomplished through the anther walls. Although these anther-wall infections have not been followed through successive stages, appressoria produced from the mycelium in the inter-anther spaces have been observed on the anther walls, and some mycelial penetration at such points has been noticed. From this stage fungous invasion of the anther tissues is very rapid. The flower buds lie so closely together in the axillary buds that usually all of the flowers from a given bud are infected. All of the anthers of an infected flower may be infected (Fig. 1).

CONTROL

During the last 3 seasons, sprays have been applied empirically for the control of stamen blight of Young dewberry, since the critical infection period was not certainly known. In 1939 and 1940, lime-sulphur, Bordeaux mixture, and a spray known as Truex (containing ammonium polysulphides) were applied at various strengths in January, February, and March. None of the sprays was effective.

Following the knowledge gained concerning the time of infection from experiments of 1941 it is the purpose of the writers to try protective sprays on axillary buds of the young canes from the period of full bloom until the berries are about half-size. However, lime-sulphur (4 per cent of 32° Baumé) applied August 20, 1941, resulted in 58.9 per cent control, as indicated by records taken May 19, 1942.

SUMMARY

A disease of the anthers of blackberry flowers is described. The causal organism, *Hapalosphaeria deformans* Sydow, infects through the anther walls and forms a pseudoparenchymatous envelope completely surrounding the pollen sac. Pycnidia formed on this pseudoparenchyma erupt on the surface ready to discharge coils of pycnidiospores when the flowers open. Infection takes place through the axillary buds sometime between May and March. No perfect stage of the causal fungus has been discovered. Lime-sulphur or Bordeaux mixture applied as a dormant spray in January, February, and March have afforded no control. Lime-sulphur applied in August has given indication of about 60 per cent control. Commercial varieties affected in Oregon are Boysen and Young dewberries and Evergreen blackberry.

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MYCORHIZAE ASSOCIATED WITH SOME COLORADO FLORA

W. D. THOMAS, JR.[1]

(Accepted for publication May 30, 1942)

In order to obtain more information concerning the mycorhizae of the Rocky Mountain region, a study was made to determine the types of mycorhizae associated with certain Colorado flora.

According to McDougall and Jacobs,[2] "Mycorhizas are not so abundant in the central Rocky Mountain forests as in some of the deciduous forests of the east. . . ." These workers found only endotrophic mycorhizae on *Pinus contorta, Juniperus monosperma,* and on three species of *Cercocarpus.* They found the ectendotrophic forms on *Pseudotsuga taxifolia* and *Populus tremuloides,* and both ectotrophic and endotrophic types on *Picea engelmanii.* Ectotrophic forms alone were found on *Abies lasiocarpa* and *Pinus flexilis.*

METHODS AND MATERIALS

The materials used in this study were collected in central and northern Colorado in the autumn of 1939 and the spring and summer of 1940. Most of the material was taken before the ground had become frozen; however, in the higher mountain regions some of the material had to be taken from frozen ground.

The roots of the specimens were taken in their entirety whenever possible. When the specimens were taken from frozen ground the entire root system was taken up with its surrounding soil and then thawed in the laboratory. The roots were killed and fixed in standard Formalin-acetic-alcohol solution, cleared, and embedded in paraffin, according to standard procedure. Sections were then stained with safranin and fast-green.

All mycorhizae found in this study were classified under the following types:

1. Endotrophic[3] (Fig. 1)
   a. Peloton—coiled masses of hyphae in the cells.
   b. Arbuscule—branched haustoria.
   c. Vesicle—"probably abortive sporangia."
2. Ectotrophic[4]
   a. Coralloid—the short-roots becoming dichotomously branched.
   b. Ball—a short, branching growth resulting in a ball composed of rootlets bound together by hyphae.

[1] The writer gratefully acknowledges his indebtedness to Dr. L. W. Durrell for suggesting this work and for helpful suggestions during the progress of the investigations. Thanks are due also to Dr. W. A. Kreutzer, Colorado State College, Dr. E. C. Stakman and Dr. C. M. Christensen, University of Minnesota, for reading and criticizing the manuscript, and to Dolores B. Thomas for assistance in preparing the camera-lucida diagrams.

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3. Ectendotrophic—a combination of endotrophic and ectotrophic types.[5]
4. Pseudomycorhiza.

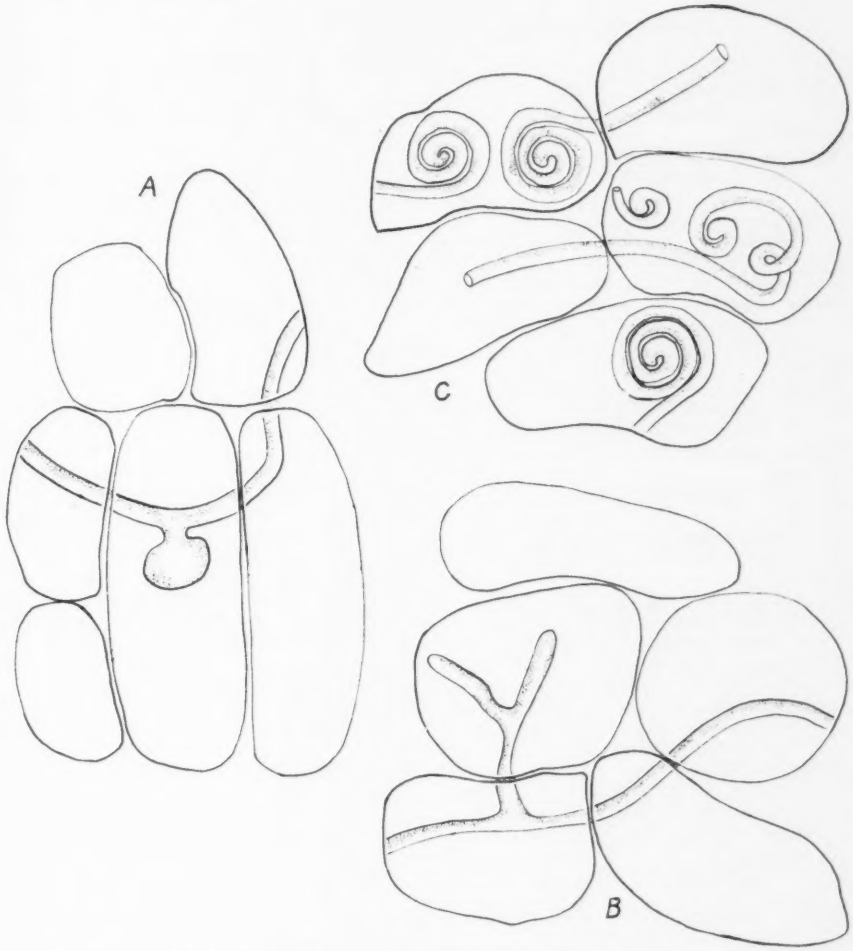

FIG. 1. Endotrophic mycorhizae. A. Vesicle in *Cirsium drumondii*. B. Arbuscule in *Potentilla gracilis*. C. Peloton in *Helianthella quinquinerva*. (All drawings made with the aid of a camera lucida.)

EXPERIMENTAL RESULTS

Ectotrophic mycorhizae appeared only on trees and shrubs, the coralloid type predominating. The ball type, with the exception of those found on *Salix scouleriana*, appeared exclusively on the members of the Betulaceae (Table 1).

Ectendotrophic mycorhizae were rare, appearing only on the roots of trees and shrubs. This type was found in only 6 species: *Picea engelmanii*,

[5] Although there is some doubt as to the ability of one fungus to produce ectendotrophic mycorhizae, this type was considered separately for convenience.

TABLE 1.—*Types of mycorhizae found on Colorado flora*

Family and specimen	Endotrophie			Ectotrophie		Ectendotrophie	Pseudomycorhiza
	Peloton	Arbuscule	Vesicle	Coralloid	Ball		
Pinaceae							
Pinus contorta Loud.	–	–	–	+	–	–	+
flexilis James	–	–	–	+	–	–	+
ponderosa Laws.	–	–	–	+	–	–	+
Picea pungens Engelm.	–	–	–	+	–	–	+
engelmanii Engelm.	–	–	–	+	–	+	+
Pseudotsuga taxifolia Britt.	–	–	–	+	–	–	+
Abies concolor Lindl. & Gord.	–	–	–	+	–	–	–
Juniperus scopulorum Sarg.	–	–	–	+	–	–	+
virginiana L.	–	–	–	+	–	–	+
communis L.	–	–	–	+	–	–	–
Salicaceae							
Populus deltoides L.	–	–	–	+	–	–	–
tremuloides Michx.	–	–	–	+	–	–	–
angustifolia James	–	–	–	–	–	–	–
Salix scouleriana Barr.	–	–	–	–	+	–	–
Fagaceae							
Quercus utahensis Rydb.	–	–	–	+	–	–	–
Ulmaceae							
Ulmus americana L.	–	–	–	+	–	–	+
Celtis occidentalis L.	–	–	–	+	–	–	–
Rosaceae							
Prunus americana Marsh.	–	–	–	+	–	–	+
pennsylvanica L.	–	–	–	+	–	–	+
virginiana L.	–	–	–	+	–	–	–
Cercocarpus montanus Raf.	–	–	–	–	–	+	–
Purshia tridentata DC.	+	–	–	–	–	–	–
Potentilla effusa Dougl.	–	–	+	–	–	–	–
fruticosa L.	–	–	+	–	–	–	–
gracilis Dougl.	–	+	+	–	–	–	–
Sorbus scopulina Britt.	–	–	–	–	–	–	–
Physocarpus monogynus (Torr.) A. Nels.	–	–	–	–	–	–	–
Rubus deliciosus James	–	–	–	–	–	–	–
Fragaria ovalis glauca (Wats.) A. Nels.	–	–	–	–	–	–	–
Geum rivale L.	–	–	–	–	–	–	–
Rosa woodsii Lindl.	–	–	–	–	–	–	–
Betulaceae							
Betula fontinalis Sarg.	–	–	–	–	+	–	–
Alnus tenuifolia Nutt.	–	–	–	–	+	–	–
Oleaceae							
Fraxinus pennsylvanica Marsh.	–	–	–	–	–	+	–
Aceraceae							
Acer glabrum Torr.	–	–	–	–	–	–	–
Leguminosae							
Robinia pseudoacacia L.	–	–	–	–	–	+	–
Gleditsia triacanthos L.	–	–	–	–	–	+	+
Thermopsis divaricarpa A. Nels.	+	–	–	–	–	–	–
Medicago sativa L.	–	–	+	–	–	–	–
Lupinus parviflorus Nutt.	–	–	–	–	–	–	–
Astragalus parryi Gray	–	–	–	–	–	–	–
Oxytropis saximontana A. Nels.	–	–	–	–	–	–	–
Polygonaceae							
Polygonum bistortoides Pursh.	+	–	+	–	–	–	–
Eriogonum alatum Torr.	–	–	–	–	–	–	–
umbellatum Torr.	–	–	–	–	–	–	–
Oxyria digyna (L.) Camptd.	–	–	–	–	–	–	–

TABLE 1.—(*Continued*)

Family and Specimen	Endotrophic			Ectotrophic		Ectendotrophic	Pseudomycorhiza
	Peloton	Arbuscule	Vesicle	Coralloid	Ball		
Polemoniaceae							
Polemonium viscosum Nutt.	+	–	–	–	–	–	–
Gentianaceae							
Gentiana elegans A. Nels.	+	–	–	–	–	–	–
parryi Engelm.	+	–	–	–	–	–	–
Swertia palustris A. Nels.	+	–	–	–	–	–	–
Frasera speciosa Griseb.	–	–	+	–	–	–	–
Scrophulariaceae							
Pedicularis groenlandica Retz.	+	–	+	–	–	–	–
Pentstemon secundiflorus Benth.	+	–	–	–	–	–	–
Pyrolaceae							
Moneses uniflora (L.) Gray	+	–	–	–	–	–	–
Primulaceae							
Dodecatheon pauciflorum (Durand) Greene	+	–	–	–	–	–	–
Labiatae							
Monarda menthaefolia Grah.	+	–	–	–	–	–	–
Saxifragaceae							
Saxifraga rhomboidea Greene	+	–	–	–	–	–	–
Heuchera bracteata Torr.	–	–	–	–	–	–	–
Ranunculaceae							
Aconitum columbianum Nutt.	+	–	–	–	–	–	–
Delphinium subalpinum (Gray) A. Nels.	+	–	–	–	–	–	–
Pulsatilla hirtissima (Pursh.) Britt.	+	–	+	–	–	–	–
Actaea arguta Nutt.	–	–	+	–	–	–	–
Ranunculus adoneus Gray	–	–	–	–	–	–	–
Orchidaceae							
Limnorchis stricta (Lindl.) Rydb.	+	–	–	–	–	–	–
Corallorhiza multiflora Nutt.	–	+	–	–	–	–	–
Spiranthes stricta (Rydb.) A. Nels.	–	–	–	–	–	–	–
Convallariaceae							
Streptopus amplexifolius (L.) DC.	–	–	–	–	–	–	–
Smilacina stellata (L.) Desf.	–	–	–	–	–	–	–
Onagraceae							
Epilobium angustifolium L.	–	–	–	–	–	–	–
Oenothera nuttalli Sweet	–	–	–	–	–	–	–
Crassulaceae							
Sedum stenopetalum Pursh.	–	–	–	–	–	–	–
Papaveraceae							
Corydalis aurea Willd.	–	–	–	–	–	–	–
Umbelliferae							
Angelica ampla A. Nels.	–	–	–	–	–	–	–
Hydrophyllaceae							
Phacelia sericea (Graham) Gray	–	–	–	–	–	–	–
Melanthaceae							
Zygadenus elegans Pursh.	–	–	–	–	–	–	–
Liliaceae							
Allium geyeri Wats.	–	–	–	–	–	–	–
Leucocrinum montanum Nutt.	–	–	–	–	–	–	–
Lilium montanum A. Nels.	–	–	–	–	–	–	–
Liliaceae							
Erythronium parviflorum (Wats.) L.	–	–	–	–	–	–	–
Yucca glauca Nutt.	–	–	–	–	–	–	–
Calochortus gunnisonii Wats.	–	–	–	–	–	–	–
Iridaceae							
Iris missouriensis Nutt.	–	–	–	–	–	–	–

TABLE 1.—(*Continued*)

Family and Specimen	Endotrophic			Ectotrophic		Etendotrophic	Pseudomycorhiza
	Peloton	Arbuscule	Vesicle	Coralloid	Ball		
Cruciferae							
Erysimum asperum DC.	–	–	–	–	–	–	–
Ericaceae							
Arctostaphylos uva ursi (L.) Spreng.	–	–	+	–	–	–	–
Berberidaceae							
Berberis aquifolium Pursh.	–	–	–	–	–	–	–
Grossulariaceae							
Ribes saximontanum E. Nels.	–	–	+	–	–	–	–
Hydrangeaceae							
Jamesia americana T. & G.	–	–	–	–	–	–	–
Campanulaceae							
Campanula parryi Gray	–	–	+	–	–	–	–
Geraniaceae							
Geranium richardsonii F. & M.	–	–	–	–	–	–	–
Monotropaceae							
Pterospora andromeda Nutt.	–	+	–	–	–	–	–
Vacciniaceae							
Vaccinium scoparium Leib.	–	–	+	–	–	–	–
oreophilum Rydb.	–	–	+	–	–	–	–
Chenopodiaceae							
Blitum capitatum L.	–	+	–	–	–	–	–
Boraginaceae							
Oreocarya virgata (Porter) Greene	–	–	+	–	–	–	–
Caprifoliaceae							
Sambucus microbotrys Rydb.	–	+	–	–	–	–	–
Linnaea americana Forbes	–	–	+	–	–	–	–
Compositae							
Helianthella quinquinervis (Hook.) Gray	+	–	–	–	–	–	–
Rudbeckia laciniata L.	+	–	–	–	–	–	–
hirta L.	–	+	–	–	–	–	–
Compositae							
Senecio crassulus Gray	–	–	–	–	–	–	–
cernuus Gray	+	–	–	–	–	–	–
eremophilus Rich.	–	–	–	–	–	–	–
Taraxacum officinale Weber	–	–	–	–	–	–	–
Chrysothamnus graveolens (Nutt.) Greene	+	–	–	–	–	–	–
Cirsium drummondii T. & G.	–	+	+	–	–	–	–
Artemisia frigida Willd.	–	–	–	–	–	–	–
Antennaria rosea (Eat.) Gr.	–	–	–	–	–	–	–
Anaphalis subalpina (Gray) Rydb.	–	–	–	–	–	–	–
Erigeron salsuginosus Gray	–	–	–	–	–	–	–
macranthus Nutt.	–	–	–	–	–	–	–
Aster engelmanii Gray	–	–	–	–	–	–	–
porteri Gray	–	–	–	–	–	–	–
Gaillardia aristata Pursh.	–	–	–	–	–	–	–
Machaeranthera varians Greene	–	–	–	–	–	–	–
Rydbergia grandiflora (Pursh.) Greene	–	–	–	–	–	–	–
Achillea millefolium L.	–	–	–	–	–	–	–

Prunus virginiana, *Fraxinus pennsylvanica*, *Robinia pseudoacacia*, *Gleditsia triacanthos*, and *Cercocarpus montanus*. It may be observed that only two of these species are widely separated from the taxonomic standpoint, whereas *Prunus* and *Cercocarpus* are in the same family, as also are *Robinia*

and *Gleditsia*. It also is interesting to note that this type of mycorhiza was not found in other members of *Picea* or *Prunus* then under observation.

Among the endotrophic mycorhizae observed, the peloton type was the most common, being the only type found in the Polemoniaceae, Pyrolaceae, Primulaceae, Labiatae, and the Saxifragaceae. The vesicular type also was common, occurring in the Salicaceae, Leguminosae, Polygonaceae, Gentianaceae, Ranunculaceae, Ericaceae, Grossulariaceae, Campanulaceae, Vacciniaceae, Caprifoliaceae, and Compositae. It may be noted that this type was consistent in its occurrence among generic groups, but was relatively inconsistent in its occurrence among family groups.

The arbuscular type, however, was somewhat rare. Arbuscules were found in *Potentilla gracilis*, *Corallorhiza multiflora*, *Pterospora andromeda*, *Blitum capitatum*, *Sambucus microbotrys*, *Rudbeckia hirta*, and *Cirsium drummondii*. It may be noticed that only in the case of *Rudbeckia hirta* and *Cirsium drummondii* were arbuscules found twice in the same family. A study of the occurrence of these various endotrophic types shows that more than one type of endotrophic mycorhiza may appear in a certain species, as was the case in *Potentilla gracilis*, *Polygonum bistortoides*, *Pedicularis groenlandica*, and *Cirsium drummondii*. No true endotrophic mycorhizae were found in the roots of trees or shrubs.

Another interesting observation was that pseudomycorhizae occurred almost exclusively on the Pinaceae, with the exception of those found on *Ulmus americana*, *Prunus americana*, *P. pennsylvanica*, *Acer glabrum*, and *Gleditsia triacanthos*.

The presence of a humus soil did not seem to control altogether the formation of mycorhizae, for mycorhizae were found on *Pinus ponderosa*, *Frasera speciosa*, and *Cirsium drummondii*, occurring in sandy soils.

It was interesting to note that all mycorhizae observed, with the exception of those in the Orchidaceae, were found among the gymnosperms and the dicotyledons. Mycorhizas were found from 5,000 to 11,500 feet above sea level, being most abundant between the elevations of 7,500 to 10,500 feet.

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LIGHTNING INJURY TO COTTON[1]

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Small areas showing lightning-injured cotton plants may be found throughout Georgia from May to September. Since 1936 the writer has been requested to examine a number of cotton fields where the growers were unable to diagnose the cause of the damage. Careful observation has indicated that such diagnostic difficulties are due to the wide variations in symptoms. These, from the standpoint of field appearance, may be grouped under two general types: (1) Well-defined, circular areas of dead plants that appear immediately or within a few days after an electrical storm. These areas have a marginal intermingling of living and dead plants (Fig. 1, A). (2) Of less frequent occurrence is a dispersed-delayed type of injury in which none or very few plants are killed outright. Usually no evidence of injury appears until ten days or more following the electrical storm. Scattered plants turn red, wilt and die (Fig. 1, B). The delayed appearance of symptoms and the absence of a well-defined central point does not readily suggest the possibility of lightning injury. Innumerable gradations between these two general types of field symptoms are encountered.

The condition that invariably appears to be associated with centralized killings is high soil moisture extending beyond the surface layer. Apparently surface water may or may not be present. High soil moisture apparently aids downward conduction of the charge and consequent killing of the underground stem, tap root, and larger lateral roots. This is followed by the sudden wilting of the above-ground parts. The downward rather than outward movement of the charge also limits the size of areas affected. The killed areas vary from 15 to 50 feet in diameter, with killed plants intermingled with the living for some distance beyond. The magnitude of the discharge obviously determines the size of the areas affected when soil resistances are similar. Areas are often elongated in the direction of the rows. The alternate furrows and ridges might result in alternate high and low soil moisture levels or streams of surface water that would tend to influence movement of the electricity along the rows. Young plants are killed outright more readily than older ones, probably because of the smaller amount of woody tissue and the nearness of the root system to the soil surface. Whipple (7) observed that higher percentages of young tomato plants were killed than of older ones. These outright killings in circular spots usually are correctly diagnosed. Consequently, the present article will be confined to the injuries that are more difficult to diagnose.

[1] Cooperative investigations between the Division of Cotton and Other Fiber Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture and the Georgia Experiment Station, Experiment, Georgia. Paper No. 95, Journal Series, Georgia Experiment Station.

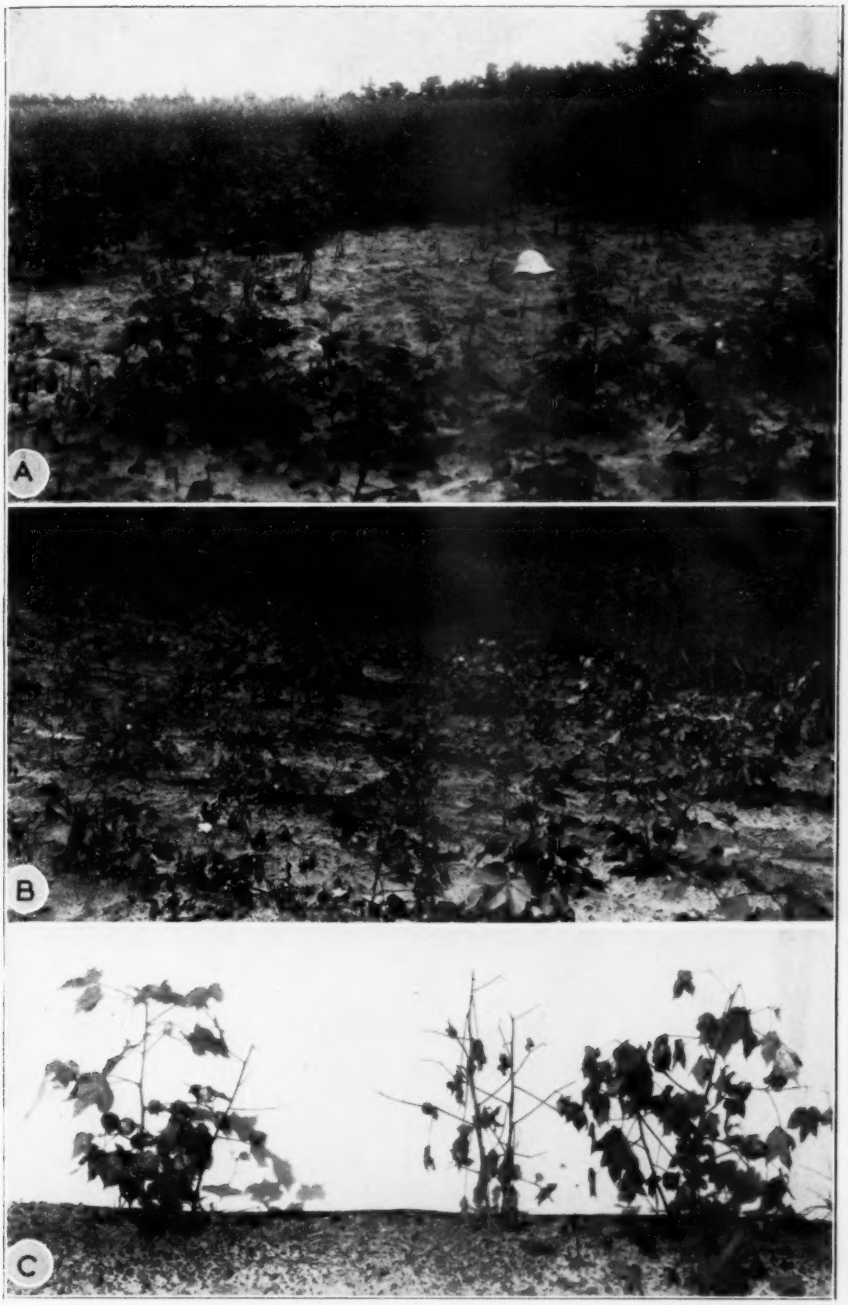

FIG. 1. Lightning-struck cotton fields. A. Typical lightning-struck spot showing denuded center with marginal killing. B. Dispersed type of injury with dead, wilting, and unaffected plants intermingled. C. Portion of cotton row in dispersed area with a dead and living plant in the same hill and adjacent hill with both plants killed.

The dispersed-delayed appearance of symptoms was called to the writer's attention in 1936, when specimens from an area approximately 200 feet in diameter were submitted to the writer by H. W. Rankin.[2] The somewhat irregularly distributed wilting and killing of plants over such a large area results in generally roughly circular spots that show no tendency to elongate with the rows. The largest spot so far observed by the writer was about 300 feet wide. Entire hills, or one or more plants from thickly clustered hills, may be affected. A typical portion of a row is shown in figure 1, C. The center of such areas usually can be determined by the higher percentage of plants showing injury; however, no well-defined center may be established for some of the larger areas. Such spots apparently result from lightning discharges that occur in relatively dry soils or in those having a thin moist surface layer with drier soil beneath. Jones and Gilbert (2) state that surface dispersion of a discharge would be expected when the first rain wets the surface soil following a period of dry weather. Where the dispersal has been so limited that a distinct "center" is discernible, diagnosis is less difficult. Plants in the immediate center of struck spots may show mechanical disorganization. Others appear to have been killed immediately through collapse of the cambium over a large portion of the plant, particularly the underground portions including the lower stem, tap root, and primary lateral roots. The pith, especially in small plants, frequently shows disorganization. This is particularly noticeable from the soil line downward; however, in the lower part of the stem the amount of pith may be so small that this character is of little diagnostic value. Further from the center direct injury to the aboveground part of the stem is infrequent. The xylem, except where actual mechanical disorganization of the plant occurs, appears unimpaired and may continue to transmit water to the upper part, even though collapse of the tissues exterior to the xylem completely girdles the stem.

The most characteristic symptom of lightning-injured plants in the delayed-dispersed type spots, and also at the periphery of those showing centralized killing, results from the passage of the charge through the soil near the surface. This produces a girdling of the stem just below the soil line and a consequent enlargement just above this girdle (Fig. 2, A). The first symptom noticeable in such plants is the reddening of the foliage. Developments subsequent to girdling are: Callus formation to produce collar-like enlargements just above the dead tissue; longitudinal or, less frequently, horizontal cracking of the dead cortical tissues; adventitious root development when the proliferated portion is in contact with moist soil; fungus invasion of the xylem at the girdle and, as invasion progresses downward, the development of discoloration and necrosis; wilting of aboveground parts because of their inadequate water supply; and eventual root starvation and death of the affected plants.

In the aboveground parts the general reddening of the foliage is attrib-

[2] Formerly Extension Plant Pathologist for Georgia.

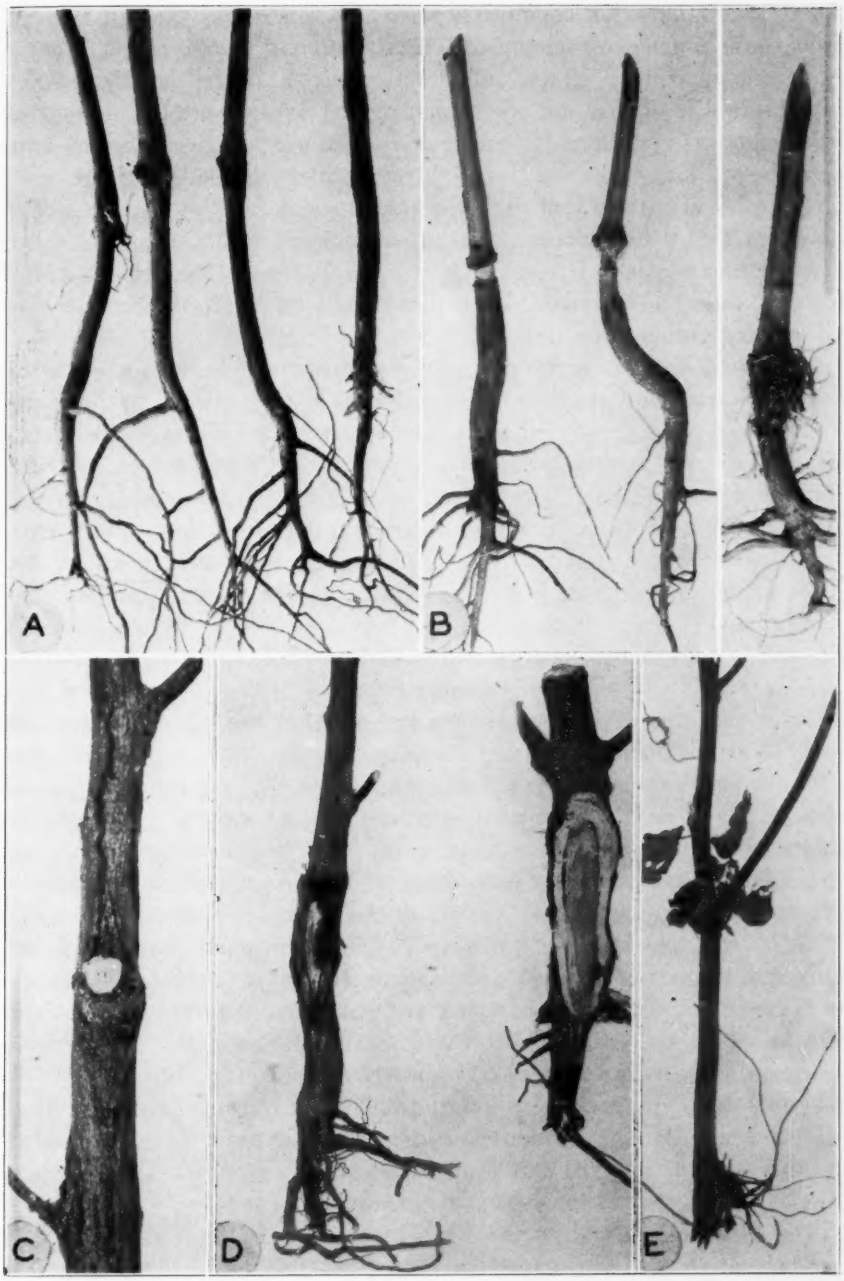

FIG. 2. Lightning-injury symptoms on cotton stems. A. Plants girdled by lightning near the soil line showing a collar-like enlargement above the injury, splitting of the dead cortical tissues, and development of adventitious roots. B. Plants girdled experimentally by removing a band of tissue below the soil line, photographed 28 days after girdling. C. Central stem of cotton plant, sometime after lightning injury, with cracking of the cortex and a wide rupture at the axis of the branch and stem. D. Incompletely girdled cotton stems which survived injury by formation of new xylem tissue when the original xylem became nonfunctional. E. Adventitious-root development sufficient for survival of the plant following complete girdling and decay of the xylem.

uted to accumulated carbohydrates, increased acidity of the cell sap, and anthocyanin pigment development. This symptom usually is followed by rather sudden wilting, death, and defoliation. That the preceding effects were the results of girdling was demonstrated experimentally in the field. Bands of tissue, ½ inch wide and exterior to the xylem, were removed just below the soil line. The resultant symptoms, including enlargements above the girdle, adventitious root development, invasion of the xylem by similar organisms, and the development of anthocyanin pigment (Fig. 2, B), were quite similar to those of lightning-injured plants. The experimentally girdled plants had survived for a period of four weeks when they were removed for examination.

Many variations in symptoms, especially among recovered plants, may be noted. Irregular elongated areas of sunken, necrotic tissue may show on the stems (Fig. 2, C), whereas the lateral branches develop normally. Wide fissures are frequently noticeable above and below the axis of these branches with the stem. The xylem, underlying the affected cortex, continues to conduct water to the uninjured branches; and their growth is affected only slightly, if any. The tops of some plants may be killed down to one of the lateral branches. The pith below the injured cortex in surviving plants is often discolored. The above-described symptoms have been observed at mid-season or later and usually are confined to the more central zone of struck spots. The infrequent occurrence of such symptoms and the irregular patterns of injured tissues suggest that the plants were wet or partly so at the time of lightning discharge.

Continued survival of girdled young plants is apparently not very common. On older plants partial girdling and recovery is more frequent. Recovered plants may appear to be normal. When, however, such plants are pulled and examined, the stems at or under the groundline are flattened, in most instances, due to a new growth of tissues on one side and the exposed xylem on the opposite side. Walker (6) has previously reported greater damage to the side of cabbage stems facing the center of the discharge area. With the plants illustrated in figure 2, D, the cortical and cambial tissues were killed on the side nearest the discharge. The xylem thus exposed became infected by organisms and eventually became non-functional. With other plants an enlargement below the soil line with much cracking of the old cortex was the only evidence of injury having occurred. Occasionally the formation of adventitious roots is adequate for support of the plant after complete decay of the stem at the injury (Fig. 2, E).

The dispersed-delayed lightning injury effect, in some instances, may have been attributed to fungus organisms (1). In fact, in the first instance that came to the writer's attention, pathogenic organisms were suspected, and tissue cultures were made from the xylem of a number of plants. *Rhizoctonia bataticola* (Taub.) Butler was obtained from a majority of the cultures and *R. solani* Kühn was second in frequency of occurrence, often in combination with the former. Other organisms were secured less fre-

quently. It was evident that *R. bataticola* entered the xylem in the region of the girdle and progressed downward into the roots and later invaded the cambium, phloem, and cortical tissues. The tissues above the girdle, supplied with evolved plant food, were not attacked by *R. bataticola*, although a portion of the vessels in this region became discolored. *R. bataticola* is of interest in connection with reports of its pathogenicity on cotton of some species other than *Gossypium hirsutum* in India (4, 5), Greece (3), and Africa (1). American upland varieties in comparison with varieties of *G. herbaceum* were found relatively resistant to this organism (3).

SUMMARY

The general appearance of lightning-struck spots in cotton fields varies considerably. In the more frequently encountered spots sudden killing in small circular areas result. More difficult to diagnose are the somewhat indefinite and variable spots in which dispersed and delayed appearance of symptoms, without noticeable centralized killing, extends over areas as much as 300 feet in diameter. Immediate killing of plants results from the collapse of tissues exterior to the xylem on the stems, tap roots and the larger roots. Plants surviving for varying periods almost invariably show a collar-like enlargement at or just below the soil line where the plants are completely or occasionally partially girdled. The girdling is caused by the killing of cortical and cambial tissues in a band that is usually about ½ inch wide. Other symptoms of surviving plants include irregular, longitudinally-elongated necrotic areas on the side of the stems. *R. bataticola*, reported to be parasitic on certain Asiatic species of cotton, frequently invades the lower stem and roots of lightning-injured plants of *G. hirsutum* in Georgia.

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DECLINE DISEASE OF RASPBERRY[1]

S. M. Zeller and A. J. Braun

(Accepted for publication May 25, 1942)

A decline disease of the Cuthbert red raspberry has been known to exist in the Pacific Northwest for a number of years. This disease has received rather intensive study, especially in Oregon, and is perhaps the same as the one that has concerned growers of raspberries in British Columbia in recent years.

The disease is manifest wherever the Cuthbert raspberry is grown in western Oregon, and becomes epidemic in districts where this crop is grown intensively, especially where there are large commercial plantings. Our studies on the decline disease have been conducted entirely in Oregon; therefore, the consideration of the occurrence is based on Oregon climatic and edaphic conditions. In some plantings where the disease is just starting the damage is slight, while in others the losses of plants vary from serious to almost total within 3 or 4 years after planting.

The economic importance of the decline disease is measured essentially by the percentage of plants affected. A plant that has had the disease for one full season is almost valueless. Relatively large diseased areas in a planting, therefore, may easily reduce yields below a profitable basis.

Although other varieties and species of *Rubus* show somewhat similar dying out of plants, the decline disease has not been proved outside of the Cuthbert raspberry.

SYMPTOMS

Unfortunately, there are no consistent or continuous symptoms by which alone the decline disease may be identified. Diagnosis depends more upon chronological performances than upon concrete symptoms. This is truly a "running-out" disease.

If infection takes place late in the season the first indication of the disease the following spring is the retarded appearance of the new succulent shoots. These have more of a reddish color than the earlier healthy canes. During the growing season, however, the leaves show no abnormalities until growth slows down in the autumn. Leaves of diseased plants produced after this time have considerable downward leaf roll and more definite fluting along the veins as compared with the flatter leaf blades on normal plants (Fig. 1). These rolled leaves toward the tips of affected canes possess somewhat less interveinal greenness than normal leaves, and are slightly bronzed along the margins and crests between the veins. The internodes in these tip areas are foreshortened, as indicated in figure 1. This condition, as suggested above, usually does not occur at other times during the growing season, under field conditions. In the greenhouse, however, this leaf-rolled condition is a predominant symptom throughout the cane-growth period

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(Fig. 3, A). Canes of affected plants, under field conditions, do not attain the height and diameter of normal growth and are otherwise weakened and unhardy, as indicated by the fact that they may die in the winter, or the buds lack the vitality to produce lateral growth the following spring. A general decrease in size and abundance of the root system parallels the depletion of the canes. The smaller roots and feeder rootlets gradually become less numerous as the disease progresses. The influence of all of these

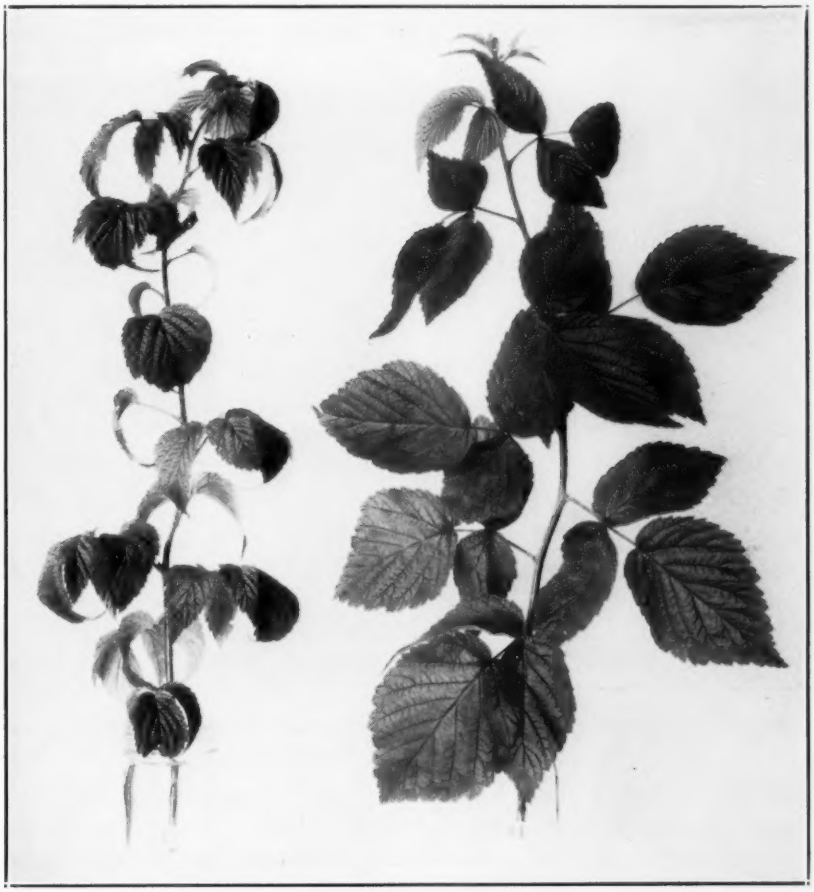

FIG. 1. Tips of current season growth of Cuthbert raspberry canes taken from the field, October 8, 1941. Cane on left infected by decline disease shows leaf rolling and fluting symptoms. Healthy cane at right.

retarding factors results in a progressive deterioration of the whole plant over a maximum of about 3 years (Fig. 3, B).

The raspberry decline disease has no leaf or cane symptoms similar to those that characterize other described virous diseases, but fruit symptoms are similar to those of the Cuthbert raspberry infected with green-mottle mosaic (Rubus virus 1) or yellow mosaic (Rubus virus 2). The fruits are

small, irregular, and tend to be globose rather than ovoid. When the ripe berries are picked the druplets fall apart readily, producing a condition commonly referred to as crumbliness. The berries, therefore, are worthless, and are not harvested from infected portions of the fields.

The disease usually forms circular areas by spreading from foci, which doubtless are single infected plants. The effects of the disease in this respect are quite similar to those resulting from root-weevil infestation. Figure 2 illustrates one of these areas, which was over 200 feet in diameter. There are usually no healthy plants left within the involved areas. This "clean

FIG. 2. Sector of a diseased area in Cuthbert raspberry planting showing damage done by the decline disease. (Photographed May 25, 1940.)

sweep" by the disease might lead to the conjecture that spread of infection involves some underground factor, such as natural root grafts or a hypogeous biotic vector. Decline disease occurs on several different soils, indicating that type of soil is probably not a factor in its epidemiology. Drainage and fertility are not factors in all localities where the disease occurs.

EXPERIMENTAL STUDIES

Previous to the grafting experiments that resulted in the transfer of the disease from affected buds or patches of bark to healthy Cuthbert raspberry plants, many types of empirical tests were made to learn something of the nature of the disease. Following unsuccessful attempts to isolate a causal organism from canes and roots, samples of soil from diseased areas were cultured by W. B. Bollen, Soil Bacteriologist, Oregon State College. No

unusual soil or parasitic organism was found. Healthy and diseased plants were grown in pots in the greenhouse in soils originating from healthy areas in the field where the decline disease was prevalent. In all such cases

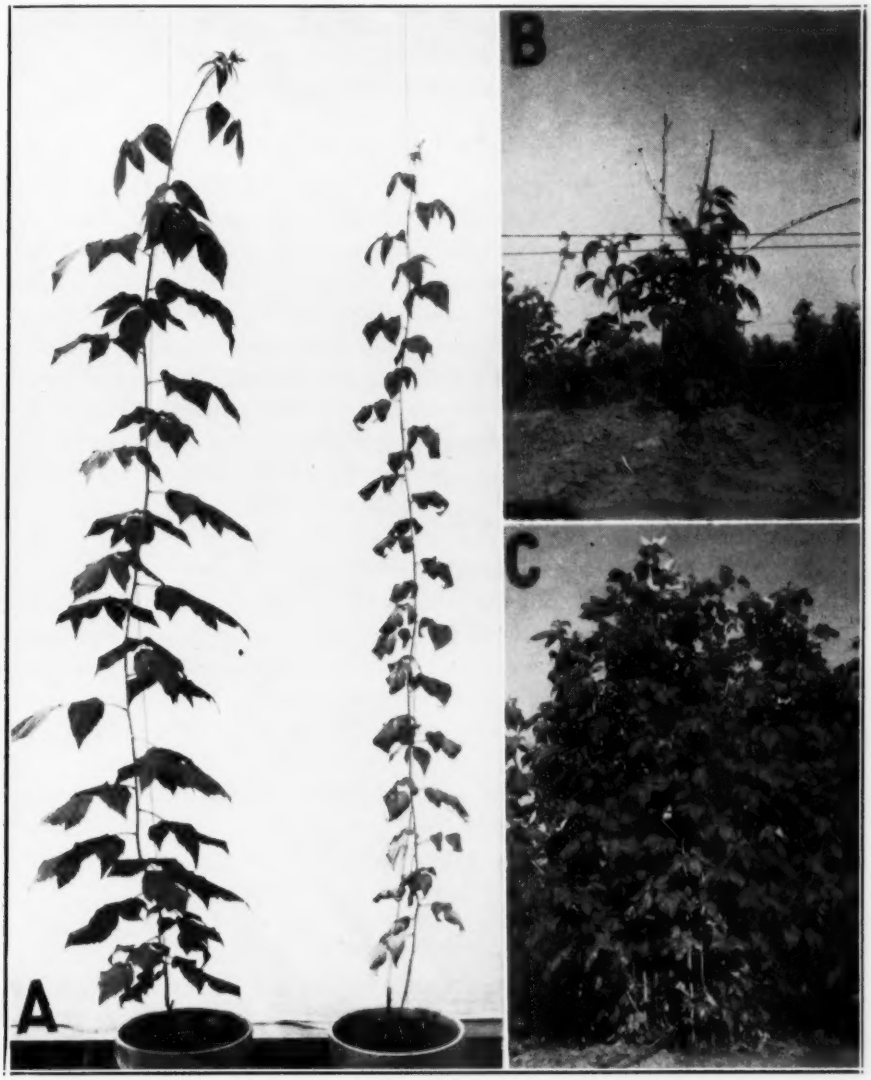

FIG. 3. A. Cuthbert raspberry plants grown in greenhouse: One at right infected by decline disease shows extreme leaf symptoms; one at left, healthy. (August 5, 1941.) B. Diseased plant photographed in the field. C. Healthy plant in same row and 50 feet from diseased plant B. (June 26, 1941.)

the healthy plants remained healthy and diseased plants developed acute symptoms (Fig. 3, A).

When a healthy and a diseased plant were grown in a single pot of soil, the healthy plant in each case has remained healthy for one year, even though

the plants became pot-bound by the intermingling of the roots of the two plants. Negative results from several such experiments indicate that natural grafts of raspberry roots in close proximity in the soil are not readily accomplished.

Good balanced fertilizers, or stable manure, added to the culture of plants with the decline disease in the field greatly stimulated their growth, but did not effect a cure or result in the eradication of the disease.

TRANSMISSION BY GRAFTING

Although under field conditions there is very little about the disease to suggest a virous etiology, we finally resorted to the grafting of roots and canes. In several cases roots of healthy plants were grafted to roots of diseased plants, buds from diseased canes were grafted into canes of healthy plants, and diseased plants were inarch-grafted to healthy plants. So far no cases of transmission through root grafts have been apparent, but in all cane-graft experiments, whether organic union between diseased cions and healthy canes was or was not accomplished, the disease was transmitted to the healthy plants. In some cases of bud grafts the diseased buds did not live, but the disease was transmitted to the healthy plants, as through "patch" grafts. All of the above grafts were made in late summer on potted plants in the greenhouse. No symptoms appeared that autumn (1941). Accordingly, these potted raspberry plants were set outside all winter and brought back into the greenhouse the next spring (1942). As soon as leaves were well developed the leaf-rolling symptoms of the decline disease characteristic of greenhouse-grown plants appeared throughout the stock plants that had been healthy the previous season. When new succulent canes appeared from the soil they bore rolled leaves, and showed all the characteristics of the disease.

It appears, therefore, that the decline disease is caused by a virus, which is readily transmitted through grafting. A total of 8 cane grafts was made. Transmission of the decline virus of *Rubus* was effected in all.

No insect vector of this virosis has been discovere

CONTROL

No specific measures have been tested for the control of the raspberry-decline disease. Incidentally, however, plant selections, made by Rowell Brothers, Scholls, Oregon, to rid Cuthbert raspberry planting stock of a bud-perpetuated disease, known locally as "crumbly berry," have resulted also in a stock that has remained free from the decline disease for several years after planting in disease-free soil. Two Cuthbert plantings within a locality where the decline disease has been epidemic have recently been certified by the Agricultural Extension Service of Oregon State College as free from all known viroses, including that now known as the decline disease. Control of the disease will, therefore, be attempted through certification of planting stock. Other varieties of raspberries and other species of *Rubus* are under test for resistance to decline.

For want of a better descriptive common name for this virosis we are using decline disease of raspberry. As a binomial we suggest **Minuor ruborum** (from *minuor*, to dwindle or decay, and *ruborum*, of *Rubus*): and Rubus virus 8 in the numerical system.

SUMMARY

A disease of Cuthbert red raspberry has caused the decline of large areas of plants in commercial plantings throughout the Willamette Valley of western Oregon. Affected plants present no particular disease symptoms, except a gradual dwindling in vigor of canes and roots, and some leaf rolling and fluting in late autumn. This leaf roll is characteristic throughout the season when affected plants are grown in the greenhouse.

This decline disease of raspberry has been transmitted by grafting diseased buds onto canes of healthy plants. No insect vector has been discovered. The common name, decline disease of raspberry, is proposed for this virosis, *Minuor ruborum* is suggested as a binomial, and Rubus virus 8 as the numerical designation.

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SOME EFFECTS OF SAND AND NUTRIENT SUPPLY ON A PHYSIOLOGICAL LEAF SPOT OF CANTALOUPE[1]

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(Accepted for publication May 30, 1942)

Attempts to grow cantaloupe (*Cucumis melo* L.), during the winter, in quartz sand with a constant supply of nutrient have not produced plants of desired vigor or quality. Plants so grown were rather spindling and lacked deep green color. The lower leaves developed water-soaked areas, which collapsed within a period of 24 to 36 hours. The leaf spotting, in severe cases, resulted in progressive defoliation of the plant until only the young leaves near the growing point remained.

Unpublished data of Crane and Shear have shown that similar troubles with tung trees grown in sand were apparently caused by lack of aeration because of the large water-holding capacity of the sand and that these troubles might be alleviated by using a coarser sand. Since cantaloupe plants are known to grow best in a well-aerated soil, it was thought that the difficulties experienced in the greenhouse may have resulted from inadequate aeration of the roots or from the method of applying the nutrient solution.

Two grades of white quartz sand were employed (Table 1). The ratio of air space in coarse sand as compared to fine sand was 8 : 1. Four methods of

TABLE 1.—*Sieve size of the two grades of sand used*

Sieve mesh	Per cent of sand passing through	
	Course "F"	Fine No. 1
	Per cent	*Per cent*
20	99.650	100.000
40	6.490	52.140
60	0.225	2.700
80	0.134	1.410
100	0.058	0.455

supplying nutrient solution were used, viz.: (1) 1000 ml. per crock, per 24 hours, drip; (2) 1000 ml. per crock, per 24 hours, slop; (3) 500 ml. per crock, per 24 hours, drip; (4) 500 ml. per crock, per 24 hours, slop. The nutrient solution used was of the following composition: 0.005 M · $Ca(NO_3)_2$, 0.0025 M · KH_2PO_4, and 0.0025 M · $MgSO_4$. This solution was supplemented with Fe, Mn, Zn, Cu, and B. Because of numerous cloudy days during mid-winter in this area, supplementary illumination was provided during the day (8 a.m. to 6 p.m.) by six 300-watt bulbs arranged on a rack suspended above the crocks. Twelve seeds of the variety White Seeded Pink Meat were sown in each crock and thinned when the first true leaves had appeared,

[1] Scientific Paper No. A28, Contribution No. 1844, Department of Botany, University of Maryland Agricultural Experiment Station.

leaving two uniform plants per crock. The sand was kept damp until emergence, at which time the nutrient treatments were started. The plants were grown for a period of 6 weeks when the various root, stem, and leaf measurements were obtained.

The data on plant growth showed that plants grown on fine sand were significantly[2] longer in stem growth, but the stems were, at the same time, significantly[2] smaller in diameter than stems from coarse sand plants. The

TABLE 2.—*Effect of sand and treatment on the occurrence of physiological spotting on cantaloupe leaves*

Treatment	Total number of leaves	Affected	Healthy	"t" value
		Per cent	*Per cent*	
Coarse sand	350	6.3	93.7	
Fine sand	383	13.1	86.9	3.14[a]
Drip culture	364	17.3	82.7	
Slop culture	369	4.6	95.4	5.50[b]
1000 ml. per 24 hours	371	14.0	86.0	
500 ml. per 24 hours	362	5.5	94.5	3.88[a]
Fine sand—drip	193	18.7	81.3	
Fine sand—slop	201	7.0	93.0	3.68[a]
Coarse sand—drip	182	10.4	89.6	
Coarse sand—slop	167	1.8	98.2	3.86[a]
Fine sand				
1000 ml.—drip	94	24.5	75.5	
1000 ml.—slop	100	13.0	87.0	2.06
500 ml.—drip	99	13.1	86.9	
500 ml.—slop	102	1.0	99.0	3.43[a]
Coarse sand				
1000 ml.—drip	92	14.1	85.9	
1000 ml.—slop	83	3.6	96.4	2.52
500 ml.—drip	89	6.7	93.3	
500 ml.—slop	84	0.0	100.0	∞
Fine sand—drip	193	23.8	76.2	
Coarse sand—drip	181	6.9	93.1	4.72[b]
Fine sand—slop	202	10.5	89.5	
Coarse sand—slop	167	1.8	98.2	7.07[b]

[a] Significant at odds of 19: 1.
[b] Significant at odds of 99: 1.

different methods of applying the nutrient solution had no effect on these measurements. In general it can be said that the plants grown in the coarse sand were a deeper green, had thicker leaves, and very closely resembled field-grown plants.

The physiological leaf spotting was significantly less on the plants which were grown on the medium providing better aeration (Table 2). This was true regardless of the comparisons made, whether between sands as a whole, between methods of applying the nutrient solution on the same sand type, or

[2] Differences significant at the one per cent level.

between methods of applying nutrient solution on the two sands (except between 1000 ml. drip and 1000 ml. slop on either fine or coarse sand).

It was concluded that, under the conditions of this experiment, the coarse sand was a superior medium to the fine sand and 500 ml. applied once per 24 hours was superior to the other nutrient applications used.

DEPARTMENT OF BOTANY,
UNIVERSITY OF MARYLAND,
COLLEGE PARK, MD.

PHYTOPATHOLOGICAL NOTES

An American Oat Disease Found in Western Anatolia.—On March 24, 1938, leaf spots on fall-sown oats were observed in the experimental field of the Plant Protection Station at Bornova, near Izmir (Smyrn), Western Anatolia. They were oblong, not sharply limited, and whitish to yellowish with a reddish border (Fig. 1). They were somewhat numerous but hardly enough so to cause much damage in that field.

Microscopic examination disclosed a fungous disease. The spores of the fungus were 2-, rarely 3-septate, hyaline, closely joined in acervuli on each

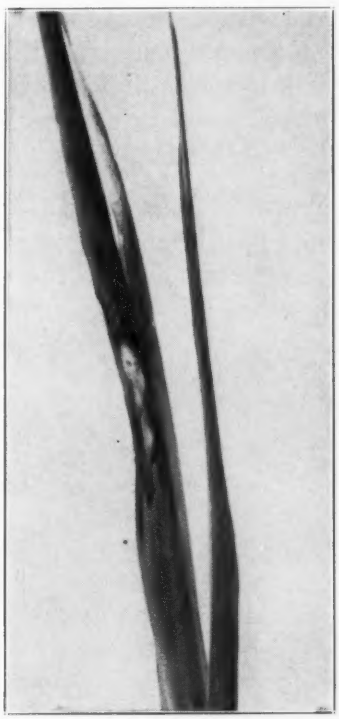

FIG. 1. Leaf spot caused by *Pseudodiscosia avenae*.

side of the leaf. They were fusiform, slightly curved spores, each bearing a single cilium at each end, the one being long and thick, the other short and slender (Fig. 2, left). Thus, they were regarded as belonging to the genus *Pseudodiscosia* Höstermann et Laubert. An end-to-end measurement of 25 conidia, disregarding their curvature, gave the following figures: length, including cilia, 23–45 μ, without cilia, 15–27 μ; average, 34.2 and 20.4 μ, respectively. The maximum width varied from 3 to 4.5 μ, with an average of 3.6 μ. The spores germinated readily in water. The disease has been, until now, unknown in Turkey, and, so far as I know, has not been observed in Europe.

Isolation of the fungus was successfully performed, but its growth was very slow on carrot agar, potato-dextrose agar, and carrot slices. On oatmeal agar and on potato sections there was no growth. In the above cultures the mycelium was hyaline, densely septate, about 3–4 μ in diameter. There was a striking tendency to form conidia of abnormal shape. This tendency was observed in cultures contaminated with bacteria and on pieces of leaves held for some time in a moist chamber, *e.g.*, in the case of increase of accompanying bacteria. The spore cells increased or decreased in number, and were swollen or abnormally slender. The cilia were swollen or their point of insertion changed (Fig. 2, right).

In reading the *Review of Applied Mycology*, I became aware of an American paper on a *Pseudodiscosia* on oats. Writing to the author of this paper, Roderick Sprague, I obtained reprints[1, 2, 3] that enabled me to identify our oat disease and prove its identity with that first observed in Oregon and

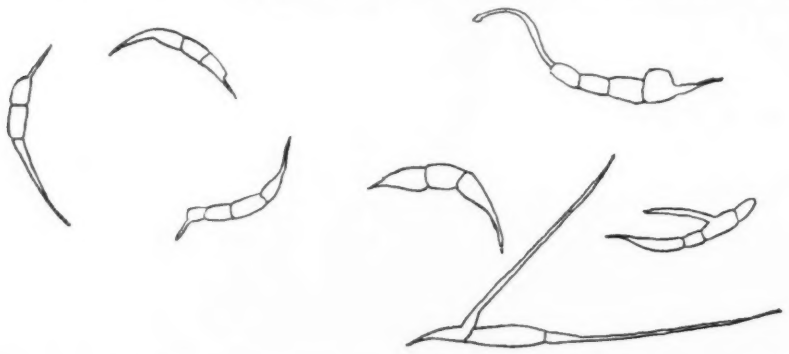

FIG. 2. Conidia of *Pseudodiscosia avenae:* normal (left) ×500; abnormal (right) ×600.

Washington in 1934. The appearance of the disease and the pathogen are the same. The conidia are of the same shape and size (10–42 × 2–4 μ), according to the American authorities. The slow and scanty development of the fungus on agar also has been observed in America. There, as well as in Turkey, the disease appears in early spring on fall-sown oats only, and disappears after the beginning of April. It seems, therefore, correlated with cool, damp weather. The writer observed it at the experimental field of Bornova in the early spring of 1939 and 1940. To date, it has caused little damage there. The American authors named the pathogen *Pseudodiscosia avenae* Sprague and Johnson.

It is a rather remarkable fact that a fungous disease of such a widegrown and frequently observed crop as oats has been observed till now solely in two countries so remote from each other as are Turkey and the Pacific Northwest of the United States. It seemed, therefore, that it might have

[1] Sprague, R. A new leaf spot on oats. Northw. Sci. **9**. 1935.

[2] Sprague, R., and A. G. Johnson. A new *Pseudodiscosia*. Mycologia **28**: 181–185. 1936.

[3] Sprague, R. Notes on diseases of cereals and grasses in Oregon and adjacent counties in Washington during the spring of 1938. Pl. Dis. Rptr. **22**: 174–175. 1938.

been imported by chance from America to Turkey; the seaport of Izmir, connected by direct steamship lines with North America, is situated not far from the experimental field of Bornova. A further observation, however, makes this assumption rather unlikely. There occurred some leaf spots of the same appearance on a wild grass on a mountain slope at Ören near Kemalpasa, at a distance of about 35 km. from Bornova. In a moist chamber there developed conidia of the same shape and size as those of *Pseudodiscosia avenae:* length, including cilia, 24–39 μ, average of 20 measurements 33.3 μ, without cilia 18–23 μ, the average being 19.9 μ, width 4–5 μ, average 4.25 μ. The grass was not in the flowering stage and, therefore, could not be determined. According to its shape, it might have been probably the wild oat *Avena sterilis* L. which species is to be found very frequently in this region. It seems more likely, therefore, that *Pseudodiscosia avenae* is a native of Turkey, and has either been imported from there to North America, or its area is very much more extended than we are aware of today.—H. BREMER, Güven Sok, No. 35, Kavaklidere, Ankara, Turkey.

Peach-suture Spot.—A new peach disease, which seems to affect only the fruit, was found last September in one Elberta orchard on the shore of Lake Ontario in Wayne County, New York. Its striking and distinctive lesions were located exclusively in the suture region, suggesting the name "suture spot" (Fig. 1.) It can be readily distinguished from the red suture

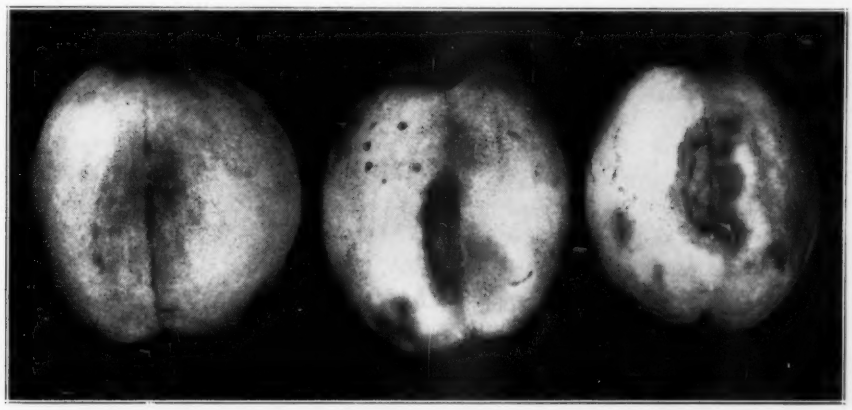

FIG. 1. Symptoms of suture spot on Elberta peach fruits at picking time. The fruit on left has a small sphere-shape lesion in the early, water-soaked stage of development, scarcely visible from the surface. The other fruits show lesions in advanced stages characterized by sunken brownish centers and deep red rims.

virosis[1] which also attacks the same part of the fruit. A similar if not identical disease is known to occur in the Niagara Peninsula of Ontario, Canada where the writer observed it during the autumn of 1940.

The most characteristic position of the lesion is astride the suture or

[1] Cation, D. Three virus diseases of the peach in Michigan. Mich. Agr. Exp. Stat. Circ. Bull. 146: 1–11. 1932.

crease line about midway between the ends of the fruit. Less frequently it may involve only one side of the suture. Only rarely are the spots out of contact with the suture or crease line and when this happens the distance of separation is only a few millimeters.

The spots are ordinarily irregularly oblong ovate in shape but in extreme cases they may be very narrow elongate or almost circular, in surface view. The largest lesions encountered occupied a maximum of one-eighth the circumference midway between the ends of the fruit and $\frac{3}{4}$ the length of to suture of the fruit. The lesions begin beneath the skin as water-soaked areas tinged with red. Lesion development proceeds first to the surface. In more advanced stages the lesions extend to the pit.

The spot lesions are characteristically red during the earlier or hydrotic stage. The centers soon change to brown upon death of the affected tissues. As the spots enlarge the outer rim becomes red, and remains level with or slightly above the fruit surface, which is in striking contrast to the brown, depressed, dehydrated, or desiccated interior of the lesion. When cut into, the texture of the affected tissues is somewhat corky and tough, with the vascular structure in prominent relief. When affected fruits with spots at different stages of development were examined, following about 6 weeks in cold storage, only some lesions of the earlier stage were found to have enlarged somewhat, and these were on fruits not fully mature.

Suture spot is peculiarly a harvest-time disease. It first attracted attention as the first fruits were beginning to ripen. By the time of the second or final picking certain trees, distributed irregularly through the orchard, had dropped a variable number of the larger more mature fruits to the ground. When it was observed that many of these fruits had striking suture-spot symptoms, it was possible to locate affected trees by looking for trees with fruit on the ground. A few trees had practically every fruit affected. Such trees might be surrounded by trees almost if not completely free of the trouble. Trees with the majority of the drops showing advanced-stage symptoms usually held less mature fruits with earlier-stage symptoms, which indicates a common relationship between symptom expression and fruit maturity. Once the fruits are mature, spot development apparently ceases.

The true cause is as yet unknown. All attempts to isolate microörganisms (bacteria and fungi) from the spot lesions have been negative, suggesting that the causal agent may be in the nature of a physiogen or virus.—E. M. HILDEBRAND, Cornell University, Ithaca, N. Y.

Additional Records of Violet Scab.—The geographic range of scab of violet (*Viola*) caused by *Sphaceloma violae* Jenkins,[1] includes the United States, new South Wales, and the Union of South Africa. Only the original record[2] of the disease in the Union of South Africa is at hand. There are

[1] Massey, L. M., and A. E. Jenkins. Violet scab caused by *Sphaceloma*. Cornell Univ., N. Y. Agr. Exp. Stat. Mem. 176. 9 pp. 1935.

[2] Jenkins, Anna E. Additional records of violet scab. Pl. Dis. Rptr. 22: 86–88. 1938.

several reports[1,3,5,6] from Australia, however, and in two instances[3,4] attention is called to its extreme destructivity to commercial violet culture. The following quotation is from the statement of 1941:

"Violet scab, which has been known to occur in this State since 1934, when it was discovered in a Richmond garden, is now common and widespread, in some seasons causing considerable loss to commercial growers. Last year, for example, in a metropolitan garden where a daily harvest of fifty dozen bunches was normally obtained, scab spoiled almost the complete crop."

In 1937 the disease had been found in 16 localities in New York, Pennsylvania, New Jersey, Maryland, Virginia, South Carolina, Georgia, Florida, and Alabama. Later it was reported[5] from the District of Columbia, on *Viola* sp., now identified as *Viola cucullata* Ait.

Violet scab occurs also in Massachusetts, Connecticut, North Carolina, Mississippi, and Texas, and in certain other localities in the States previously named. Among the new violet suscepts represented by the more recent records of the disease are *Viola jooi*, *V. priceana*, or *V. vilmoriniana*. The disease had previously been reported on *Viola cucullata?* but heretofore not on plants definitely identified as this species.[6] It was first observed on English violet (*V. odorata* L.), but until now it has not been recorded on the white-flowered variety. *V. cucullata* is a native of northeastern and *V. priceana* of southeastern United States; *V. jooi* is from southeastern Europe, and *V. vilmoriniana* from France. In transmitting the specimen of *V. jooi* Professor Whetzel wrote:

"This interesting and valuable species is one of the most beautiful in my garden. It flowers abundantly in spring before the leaves come out and again in autumn when cool weather comes on. It was recently discussed by R. T. Clausen in 'Gentes Herbarum' (4: 315. 1940)."—ANNA E. JENKINS, Bureau of Plant Industry, Washington, D. C.

Physiologic Races in Urocystis tritici.[1]—Physiologic specialization in *Urocystis tritici* Koern. apparently has not been extensively investigated. Verwoerd[2] obtained no evidence of physiologic races among several collections tested by him in South Africa, including a collection from the United States. On the other hand, Yu, *et al.*[3] described 5 races occurring in China.

[3] Anonymous. Violet Scab. Agr. Gaz. N. S. W. **52**: 103. 1941.

[4] Noble, R. J. Australia: Notes on plant diseases recorded in New South Wales for the year ending 30th June, 1938. Internat. Jour. Pl. Protect. **13**: 25M–26M. 1939.

[5] Jenkins, Anna E. Unusual collections of destructive fungi on plantain and violet in the District of Columbia. Pl. Dis. Rptr. **24**: 370–372. 1940.

[6] Compare footnote 1, p. 7.

[1] Cooperative investigations between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Agricultural Research Administration, U. S. Department of Agriculture, and the Washington Agricultural Experiment Station.

[2] Verwoerd, Len. The biology, parasitism, and control of *Urocystis tritici* Koern., the causal organism of flag smut in wheat (*Triticum* spp.) and recording the occurrence of *Urocystis occulta* (Wallr.) Rab., in South Africa as the cause of "stem smut" in rye. Union of So. Africa Dept. Agr. Science Bull. 76, 59 pp. 1929.

[3] Yu, T. F., L. Hwang, and C. T. Tsiang. Varietal resistance and susceptibility of wheats to flag smut (*Urocystis tritici* Koern.). III. Physiologic specialization in *Urocystis tritici* Koern. Chinese Bot. Soc. Bull. 2: 111–113. 1936 [Rev. Appl. Mycol. **16**: 305. 1936].

The identity of these was based on the results obtained from 4 years' tests on 10 wheat varieties. No other evidence of specialization in this fungus has been reported.

Although the occurrence of flag smut of wheat in the United States was first reported in 1919,[4] its distribution has been confined, until recently, to certain areas in Illinois, Kansas and Missouri. It was observed in 1940 near Goldendale, Washington,[5] and was apparently more widely present in 1941.[6] Investigations were begun to determine whether this smut in these two widely separated regions was caused by the same or different physiologic races.

Two collections of *Urocystis tritici* were used, one from Federation and certain other spring wheat varieties grown in the greenhouse at Arlington Farm, Virginia (originally from Leavenworth, Kans.), and the other a composite of spores from Hymar (C. I. 11605), Rex (C. I. 11689), and Hybrid 128 (C. I. 4512), winter wheat varieties, collected in 1941 near Goldendale, Wash. The inoculum was prepared by grinding smutted plant parts in a hand sausage mill and then separating the powdered spore material from the coarser plant parts with the aid of a soil sieve.

Seed of more than 100 varieties of winter and spring wheat was inoculated by shaking it and spores together in a glass vial or Petri dish. It was then planted in the greenhouse at Pullman, Wash. (1941 and 1942), Arlington Farm, Virginia (1941), and Beltsville, Maryland (1942). During the period of preemergence the temperature was maintained at approximately 15°–20° C. At Pullman, following complete emergence of the seedlings, Semesan solution was applied at intervals of 3 or 4 days for about 2 weeks in order to prevent excessive loss of plants from seedling blight.

Smut percentages were based on plant counts. At Pullman, the smutted plants were removed from the rows as the smut became evident. At Arlington Farm and Beltsville, the final counts were made when the normal plants had reached maturity. Both completely and partly smutted plants were included, and recorded separately, in the counts of smutted plants. Plants were considered smut-free if no infection was evident by heading time, although in certain varieties it was observed that smutted late tillers occasionally were produced by apparently smut-free plants. Varieties of this type, however, were not used for race differentiation.

These tests proved that the 2 collections of *Urocystis tritici* used represent 2 distinct physiologic races. This is clearly indicated by the reaction of the varieties listed in Table 1. The race from the Kansas smut is designated as race 1, and that from the Washington smut as race 2.

Certain varieties not listed in table 1 also exhibited differential reactions to these races; still others were resistant to both races. It is notable that differentiation of the two races is exhibited by resistance to race 1 and sus-

[4] Humphrey, H. B., and A. G. Johnson. Take-all and flag smut: Two diseases new to the United States. U. S. Dept. Agr. Farmers' Bull. 1063, 8 pp., 1919.

[5] Heald, F. D., and C. S. Holton. Flag smut of wheat found in Washington. Plant Dis. Rptr. **24**: 382. 1940.

[6] Holton, C. S. Flag smut of wheat in Washington. Plant Dis. Rptr. **25**: 335–336. 1941.

TABLE 1.—*Percentage of smutted plants in 6 varieties of wheat inoculated with 2 races of Urocystis tritici at Pullman, Wash., Arlington Farm, Va., and Beltsville, Md., 1941 and 1942*

Variety	C.I. No.	Place; date tested	Plants from seed inoculated with			
			Race 1		Race 2	
			Total	Smutted	Total	Smutted
			Number	*Per cent*	*Number*	*Per cent*
Federation	4734	Pullman, 1941	10	100	24	63
		" '41–'42	19	100	21	90
		" 1942	17	76	17	65
		Arlington, 1941	37	60	40	73
		Beltsville, 1942	50	46	48	69
Oro × Federation-1	11914	Pullman, '41–'42	17	82	21	86
		" 1942	17	82	18	83
		Beltsville, 1942	47	60	45	44
Oro × Federation-38	.....	Pullman, 1941	35	0	34	79
		" '41–'42	22	0	21	86
		" 1942	22	0	13	69
		Beltsville, 1942	40	0	51	59
Oro × Federation-40	.....	Pullman, 1941	17	0	31	78
		" '41–'42	23	0	22	73
		" 1942	22	0	21	29
		Beltsville, 1942	50	0	45	42
Oro × Federation-26	.....	Pullman, 1941	16	25	34	21
		" '41–'42	17	65	19	100
		" 1942	24	29	18	83
Baart	1697	Pullman, 1941	13	15	30	80
		" '41–'42	20	10	21	62
		" 1942	19	16	17	59
		Arlington, 1941	51	16	47	9
		Beltsville, 1942	48	4	44	36

ceptibility to race 2, whereas the reverse reaction has not been observed on any varieties tested.

It may be added, also, that while the reaction of Oro to the 2 races has not been tested under parallel conditions, it has been found susceptible to the Washington smut under greenhouse conditions and highly resistant to or immune from the Kansas smut, both in the greenhouse and in the field. Thus, apparently, the selections Oro × Federation-1 and Oro × Federation-26 are segregates that have the Federation type of susceptibility to both races, while Oro × Federation-38 and Oro × Federation-40 have the Oro type of reaction, *i.e.*, resistance to race 1 and susceptibility to race 2.—C. S. HOLTON and A. G. JOHNSON, Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

Cultural Differences Among Single Basidiospore Isolates of Rhizoctonia solani.—While many strains of *Rhizoctonia solani* Kühn differing in cultural characters and pathogenicity have been reported, little evidence exists as to their origin. Müller[1] has reported that some single basidiospore isolates from a basidial mat differed in cultural characters and pathogenicity.

[1] Müller, K. O. Untersuchungen zur Entwickelungsgeschichte und Biologie von *Hypochnus solani* P.u.D. (*Rhizoctonia solani* K.). Arb. Biol. Reichsanst. Land-u. Forstw. **13**: 197–262. 1924.

TABLE 1.—*Cultural strains secured from single basidiospore isolates of Rhizoctonia solani*

Basidial mat	Host	No. cultures isolated	No. cultures compared in plates	No. cultural types
A	Lima bean	7	6	6
B	" "	24	21	21
C	" "	52	22	22
D	" "	83	24	24
E	Alligator weed	60	13	13
F	" "	30	19	19
G	Irish potato	57	12	12
H	" "	4	4	4
I	" "	3	3	3
J	" "	75	29	29
Total		395	153	153

Ryker and Exner[2] stated that some variation occurred in cultures from basidiospores found in nature.

An excellent opportunity developed in the summer of 1942 to make a more careful study of single basidiospore cultures. Basidial mats were very abundant on Lima bean, Irish potato, and alligator weed (*Alternanthera phylloxeroides*); and 395 single basidiospore isolates were obtained from 10 mats. Cultures from the same mat differed so strikingly it was felt the results were of sufficient value to report.

The cultures were first compared in tubes of potato-dextrose agar. The various types found among the isolates from each mat were then compared one or more times in triplicate Petri plates of potato-dextrose agar at 28° C. The rate of growth was determined from the 24th to the 48th hour.

In table 1 there have been brought together the data concerning the number of isolates secured from each mat, and the number of culturally distinct types obtained from each mat. It is noted that as many as 29 distinct cultural types were obtained from a basidial mat. These differed in the size, shape, number, and arrangement of the sclerotia, color of the mycelium, topography of the cultures, and rate of growth. Figure 1, A, shows

TABLE 2.—*Growth in millimeters, from the 24th to the 48th hour, of 21 cultures of Rhizoctonia solani from mat B on Lima bean (av. of two experiments)*

Culture	Rate of growth	Culture	Rate of growth	Culture	Rate of growth	Culture	Rate of growth
B–1	31.3	B– 6	33.2	B–11	32.7	B–17	28.2
B–2	35.7	B– 7	32.2	B–12	31.5	B–19	28.8
B–3	33.0	B– 8	32.2	B–14	28.5	B–20	36.2
B–4	27.0	B– 9	27.7	B–15	36.0	B–21	26.3
B–5	35.7	B–10	31.2	B–16	28.8	B–22	37.2
						B–23	31.8

Difference necessary for significance at 5% pt. = 2.0.
1% pt. = 3.8.

[2] Ryker, T. C., and Beatrice Exner. A comparative study of four species of Rhizoctonia. (Abstract) Phytopath. 32: 8. 1942.

6 cultures from the same mat, which differed in cultural characters, and figure 1, B, shows that duplicate plates of the same culture were very simi-

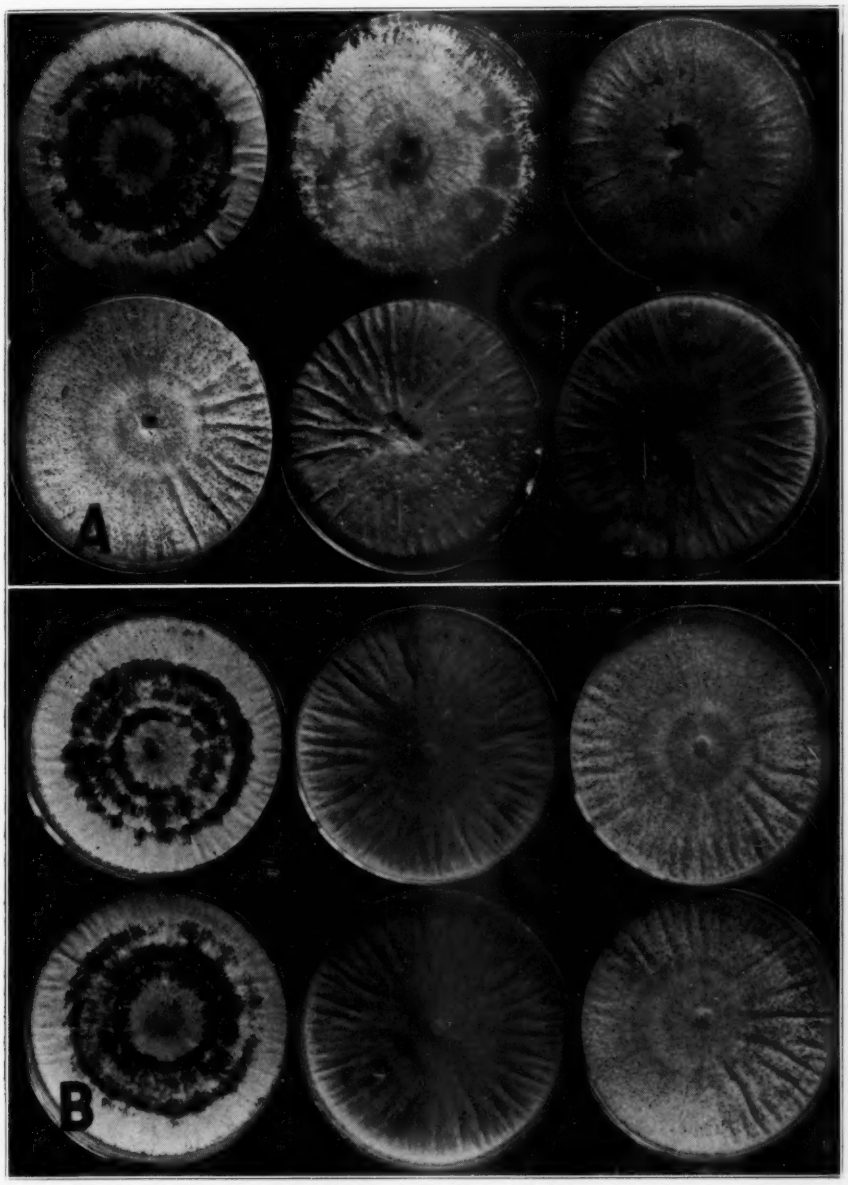

FIG. 1. A. Six cultural types of *Rhizoctonia solani* from mat D. B, Duplicate plates of 3 cultures of *Rhizoctonia solani* from mat D.

lar. Table 2, which gives the results of two experiments on the rate of growth of cultures secured from mat B, shows that the cultures differed

significantly in this respect. Although the data are not given, differences were found in growth rate among the isolates from the other 9 mats.

These results indicate that some type of segregation occurred in the formation of basidiospores and that different strains of *Rhizoctonia solani* arose as a result. Whether these cultures differ also in pathogenicity is being studied.—BEATRICE EXNER and S. J. P. CHILTON, Department of Plant Pathology, Louisiana Agricultural Experiment Station, Baton Rouge, La.